Isolation and Characterisation of Phages Infecting Gram Positive Food Bacteria

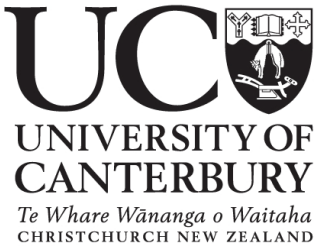
A thesis submitted in partial fulfilment

of the requirement for the

Degree of Master of Science

by

Wan-Jing Lee



School of Biological Sciences

University of Canterbury

2008

Abstract

Bacteriophage (phage), virus of bacteria, has been proposed as a mean to inactivate bacteria that are pathogens of humans. Applied prophylatically to food, phage might decrease the numbers of potential pathogens we ingest. Much active research on using the phages of bacteria to control Gram negative foodborne pathogens are described in the literatures, but comparatively little research describes the phages of Gram positive bacteria and their use as biocontrol agents on food. In this work, previous undescribed phages, able to infect *Bacillus cereus* and *Listeria monocytogenes,* were isolated from soil and ruminants faecal material, respectively. As the first step in assessing their potential as biocontrol agents, the isolated phages were purified, concentrated and characterised (albeit to different degrees). The *Bacillus* phages had a narrow host range while the *Listeria* phages had a broad host range. *Listeria* phages also infected *L. monocytogenes* 2000/47, a strain which recurs in New Zealand clinical cases. Both *Bacillus* and *Listeria* phages appeared to be of the *Myoviridae* family judging by their structure in electron micrographs. The *Bacillus* FWLBc1 and FWLBc2 phages were lytic phages with a latent period of 106 and 102 min at 37°C, and an average burst size of 322 and 300 phages per infected cell, respectively. Moreover, they both had genomes of approximately 134 kb. All newly isolated and characterized phages were chloroform resistant and survived storage better at 4°C than at room or freezing temperatures. *Bacillus* phages significantly reduced the bacterial population in mashed potatoes within 24 h at room temperature, when applied at a phage to host ratio of 1000. *Listeria* phages rapidly inactivated the host population to a low optical density. The findings of this thesis will add to the current knowledge of phages in the context of various environmental conditions for different bacteria and will demonstrate the potential of phages as food safety biocontrol agents.

List of Tables

List of Figures

Chapter 1

Introduction

1.1 General Introduction

Current technologies employed to inactivate bacterial pathogens on foods are not infallible which eventuality leaves room for new approaches for improving food safety. Moreover, indications are that consumers favour food with fewer chemical preservatives (Daeschel, 1993) because they are aware of the potential health risks associated with some chemical agents, making the use of bacteriophage (phage) as a biocontrol of potential pathogens an attractive option. Biocontrol using phage, a “phage therapy on food”, has been proposed as a promising alternative method, which involved the use of naturally occurring viruses that infect and kill the bacteria in the food (Levin and Bull, 1996; Goodridge and Abedon, 2003; Alisky et al., 1998).

Many studies on biocontrol using phage have been done on Gram negative bacteria that are human pathogens (i.e. *Escherichia coli, Campylobacter jejuni, Salmonella*) (O’Flynn et al., 2004; Bigwood et al., 2008; Carey-Smith, 2006; Atterbury et al., 2003b). However, comparatively little work has been done on biocontrol using the phages that infect the Gram positive bacteria often found in food. Phage biocontol of Gram positive pathogens such as *Listeria* and *Bacillus* have not been the subject of extensive testing. *Listeria monocytogenes* being the most common strain in *Listeria* species that are associated with foodborne illness whereas *Bacillus cereus* is a spore-forming bacterium whose spores survives most cooking processes and can be readily isolated from a variety of foods. In this thesis, the focus will be on these two bacteria and associated phages. The use of phages as biocontrol agents is not simple because the phage-host interaction is complex with many different interactions

between phage and host during the course of an infection. The background information on phages and the targeted bacteria, *B. cereus* and *L. monocytogenes*, is covered in this chapter as background for the details of the experimental approaches taken and described in later chapters.

1.2 Bacteriophages

Phages are abundant in the environment and are suggested to have likely been infecting bacteria since they first evolved (Kennedy and Bitton, 1987; Thiel, 2004). Phages were first discovered during the second decade of the 20th century (Twort and Lond, 1915; Goodridge and Abedon, 2003) and the bactericidal activity of phages was first observed by Hankin in 1896 (Sulakvelidze et al., 2001). Hankin noticed that filtered water from the Ganges and Jumna rivers in India had antibacterial properties against *Vibrio* C*holerae*. Twort, Lond at 1915 and d'Herelle at 1917 then study the characteristics of this filterable and transmissible agent independently (Twort and Lond, 1915; d'Herelle, 1917). Due to the agent's ability to kill bacteria, d'Herelle named the agent 'phage' from the Greek word '*phagin*', meaning "to devour or eat" (d'Herelle, 1917). Although the initial isolation of phage offered the first therapy for controlling infectious disease, however, the development of "phage therapy" against bacterial diseases was affected by the discovery of antibiotics in the 1940s (Goodridge and Abedon, 2003; Hudson et al., 2005; Marks and Sharp, 2000), which yielded better results for controlling human disease at that time. Contributing factors that lead to the eclipse of phage therapy development included poor understanding of mechanisms of bacterial pathogenesis and the nature of phage-host interactions in earlier time (Hudson et al., 2005; Marks and Sharp, 2000; Goodridge and Abedon, 2003; Walker, 2006).

With the increase in multiple drug-resistant bacteria, phages are now being re-evaluated as a source of new therapeutic agents. In the 1980s, Smith and co-workers demonstrated that

results of phage inactivated *E. coli* in mice proved to be effective even when compared to antibiotics. Their finding renewed the interest in exploring the use of phages as biocontrol agents (Smith and Huggins, 1980; Smith and Huggins, 1982; Smith and Huggins, 1987; Smith and Huggins, 1983, Smith et al., 1987). Over the last 20 years, phage researchers have accumulated much more relevant information about the use of phage as therapeutics.

1.3 Introduction of phages

1.3.1 Characterisation of phages

Typically, phages consist of an outer protein capsid enclosing nucleic acid genomes. The genomes of phages can be either linear or circular molecules of double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), or single-stranded RNA (ssRNA) between 5 and 500 kb pairs long (Goodridge and Abedon, 2003). Phage can vary in many different sizes and shapes with the structure varying from complex polyhedral capsid with a tail and tail fibers to a comparatively simple polyhedral capsid (Ackermann, 2007). Most (i.e. 96%) of phages described to have tails and appears to be the oldest known virus group (Ackermann, 2003). Morphology, along with the nature and size of phage genome, are used in the classification of phages (Table 1.1).

1.3.2 Lifecycle and Mechanism of the Activity of Phages

Most phages fall into two groups, virulent or temperate. The characteristic lifecycles, lytic and lysogenic cycles, of phage is illustrated in Figure 1.1. Virulent phages go through a lytic cycle, which can release up to several hundred progeny per cell. The lytic cycle, while underlain by copious variety and detail, basically involves adsorption, infection, replication and release of progeny phage.

Table 1.1 Classification of phages by Morphology and Genome (McGrath, and van Sinderen, 2007; Ackermann, 2003; van Regenmortel et al., 2000; Krylov, 2001; Sharp, 2001)

Family	**Morphology**	**Nucleic Acid Strands**	**Nucleic Acid Type**	**Genome Size kbp or kb**	**Genome arrangement**
Myoviridae	Non-enveloped Contractile tail	2	DNA	39-169	Linear
Siphoviridae	Non-enveloped Long Non-contractile tail	2	DNA	22-121	Linear
Podoviridae	Non-enveloped Short Non-contractile tail	2	DNA	19-44	Linear
Tectiviridae	Non-enveloped Isometric	2	DNA	15	Linear
Corticoviridae	Non-enveloped Isometric	2	DNA	9	Circular
Lipothrixviridae	Enveloped Rod-shaped	2	DNA	16	Linear
Plasmaviridae	Enveloped Pleomorphic	2	DNA	12	Circular
Reoviridae	Non-enveloped Isometric	2	RNA	1-4	Segmented
Fuseloviridae	Non-enveloped	2	DNA	15	Circular

	Lemon-shaped				
Inoviridae	Non-enveloped Filamentous	1	DNA	7-9	Circular
Microviridae	Non-enveloped Isometric	1	DNA	4-6	Linear
Leviviridae	Non-enveloped Isometric	1	RNA	3-4	Linear
Cystoviridae	Enveloped Spherical	2	RNA	13	Segmented

The first step of phage attachment is called adsorption. Adsorption is a diffusion limited process (Molineux, 2001) that relies on proteins that are on the phage surface to attach to the specific receptors on bacterial cell surface (Downes and Ito, 2001). More than one phage can be adsorbed to a single bacterium simultaneously. In the case of tailed phage, the particle encounters and attaches to specific bacterial surface receptors is on the tip of its tail. In the case of phages without tails, other appendages, such as protein spikes, are used for the attachment to receptors on the bacterial surface (Sharp, 2001). This adsorption process does not require energy (in most cases) and thus can occur at low temperatures. Hence, phages can be dissociated from the bacterial cell at this initial step of adsorption (Hancock and Braun, 1975; Said et al., 2003). Studies have shown that the concentration of ions in the solution, especially calcium and magnesium (Downes and Ito, 2001), plays an important role on the effect of electrostatic bonds between phage and their receptors during adsorption, therefore, influence the rate of phage attachment in most of the cases (Downes and Ito, 2001).

Under optimal conditions, adsorption becomes irreversible and secondary energy (possibly ATP) (Prescott, 1993) is required for phage genomes to enter the host cell and into the

bacterial cytoplasm, which leads to phage protein capsid being left at outside of the host cell (Abedon, 2002; Molineux, 2001). In the case of tailed phage, the tail sheath contracts and the core is driven through the wall to the membrane of bacterial cell. This process is called penetration or injection. The injection process may be both mechanical and enzymatic with examples of *B. subtilis* phage Ø29 and *E. coli* T7 phage respectively (González-Huici et al., 2004; Molineux, 2001).

Immediately after injection of the viral genome there is a stage of replication. Replication stage includes a process of synthesis of phage nucleic acid and proteins. The host's normal synthesis of protein and nucleic acids is disrupted, and is forced to undergo synthesis of viral products at this stage. Having replicated all of the virion parts, there follows assembly and packaging process. In most but not all cases, phage encoded enzymes (Hudson et al., 2005) which rupture the cell wall of bacterial cells. This leads to lysis and killing of the bacterial cell, with releasing of progeny phages (with the well known exception of M13 phage) (Marvin, 1998). Enzymes such as holins (Young and Bliisi, 1995; Young et al., 2000), endolysine (Young et al., 2000;), and lysozyme (Thiel, 2004) are produced as late viral proteins and are used to assist the phage progeny to lysis the cell wall peptiodglycan from the inside of bacterial cell. The released of mature phages then spread to nearby bacterial cells and complete the cycle. Virulent phages normally produce clear plaques in lawns of susceptible bacteria, which results from the lysis of bacteria (Marks and Sharp, 2000).

Although temperate phages are able to kill bacteria through a lytic cycle, they can alternatively persist as prophages in the lysogenic cycle. After the adsorption and injection stages, the temperate phage genomes enter a quiescent state in the bacterium where most of the phage genes are not transcribed (Adams, 1959; McGrath and Sinderen, 2007) in contrast to the lytic cycle. The phage genome in this repressed state is called a "prophage" (Purves et

al., 1995; Marks and Sharp, 2000; Goodridge and Abedon, 2003). In most but not all cases, the temperate phage's genome actually integrates into a host genome and is replicated along with the chromosome and passed on to daughters during cell division. A bacterium that harbours a prophage is termed a "lysogen" (Murray et al., 1998). Even though the lysogenic state is very stable, a temperate phage is also able to switch to a typically lytic life cycle, where the prophage is activated. The switching from lysogenic to lytic cycle can be in response to an external signal, although the switching mechanisms are poorly understood in most cases (D'Ari, 1985). Temperate phages are usually observed as turbid plaques on lawns of susceptible host cells (Marks and Sharp, 2000).

Apart from the two lifecycles described above, Ripp and Miller proposed a special condition called pseudolysogeny (or the unstable carrier-state). Pseudolysogeny is a condition where phage genomes behave like a prophage regardless it is a virulent or temperate phage (Ripp and Miller, 1998). Under stressful habitats, phage genomes coexist in an unstable relationship with bacterial cells for extended periods. This condition is a type of suspended animation which results from a low energy state caused by factor such as concentration of nutrients available, presence of antibiotics in the bacterial cell or at low temperatures in the bacterium (Ripp and Miller, 1998). Hence, the metabolism of the bacterium is slow and generation times increased. Nevertheless, once the nutrients are added or stressful habitats are removed from the bacterium, the pseudolysogens resolves and enter into either true lysogenic cycle or lytic cycle (Ripp and Miller, 1998).

The phenomenon of "lysis from without" (LWO) was described by Delbrück and Ellis (Delbrück and Ellis, 1939). LWO can take place when a sufficiently high number of phage particles adsorbed on to a bacterium and lysis through activity of cell wall by degrading enzymes (Watson, 1950). However, in contrast to phages undergo the lytic cycle, no active

phage is produced through this type of “lysis” and the cell does not demonstrate the cytochemical differences evident after phage infection (Watson, 1950; Luria and Human, 1952).

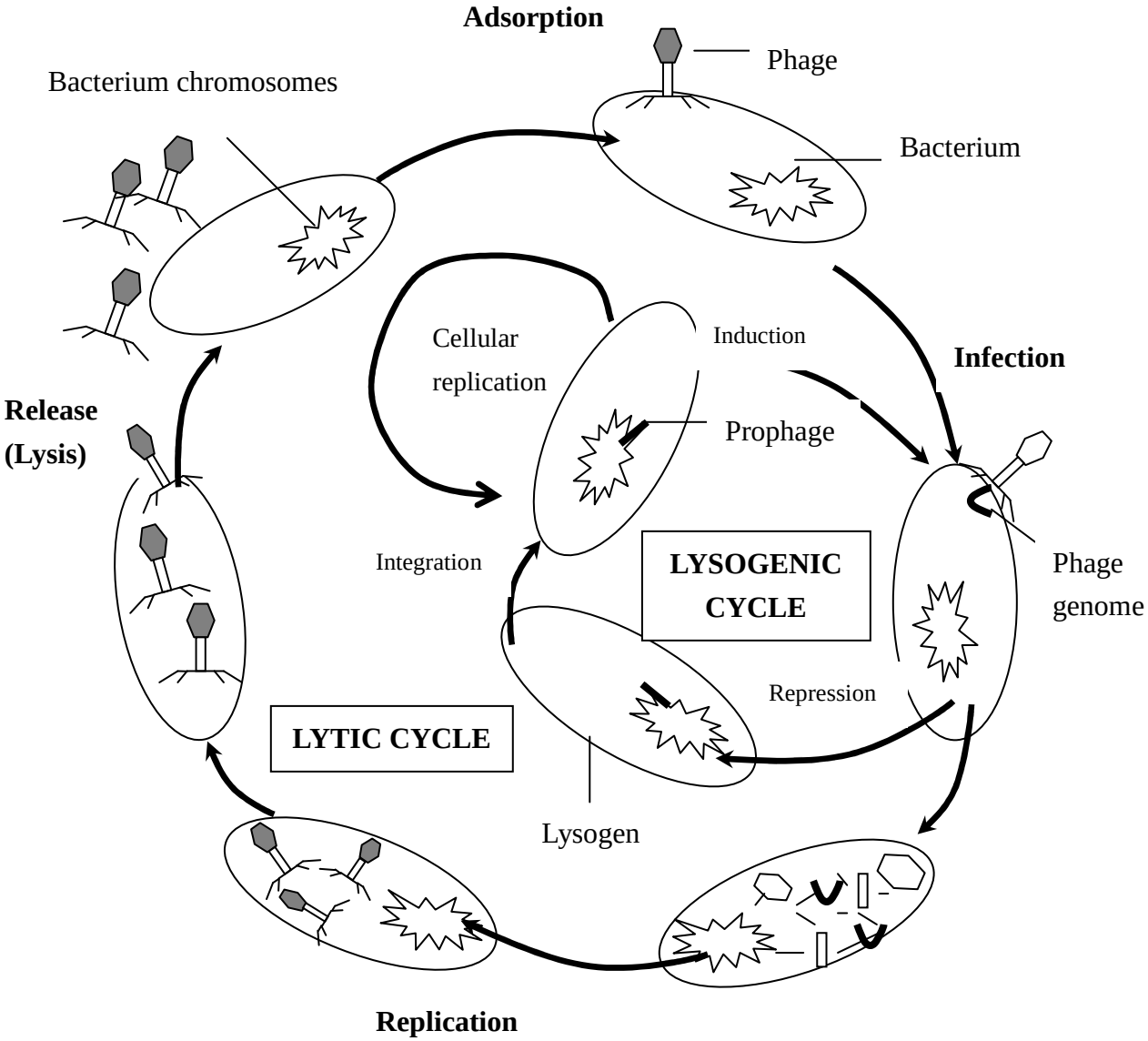


Figure 1.1 Diagram representing the lifecycles, lytic and lysogenic cycle, of phage. This shows the free phage (phage unattached to a bacterium) finding an attachment site on a bacterial cell surface. Follows deposits its genetic genomes into the bacterial cell and goes under lysogenic or lytic cycle for replication.

1.4 The Need for Therapeutic Agents Against Bacterial Pathogens

An increase in antibiotic resistant bacteria has now become a global issue and there is a need for a new and effective method to control the pathogens (Barrow and Soothill, 1997; Reacher et al., 2000; Goossens et al., 2005). A potential alternative to antibiotics that has been proposed is phage therapy, which would involve the application of phages to bacteria-containing ecosystems to reduce deleterious bacterial populations. The main and obvious inherent advantages of phage therapy over antibiotics are their abundance in the environment and its specificity characteristic toward bacterial cells (Goodridge and Abedon, 2003). The ubiquity and diversity of phages in the environment means that numerous alternative phages, differencing in host ranges, which attack different diversity of bacterial cells are already supplied in the environment (Goodridge and Abedon, 2003). Although phages resistance in bacteria are likely to arise, Inal suggested that this should not be a major concern in comparison to antibiotic resistance (Inal, 2003). It was explained that phage being a living organism, will co-evolve with their host bacteria as they developing resistant against particular phage (Inal, 2003; Carlton, 1999; Goodridge and Abedon, 2003). The high specificity of phages ensure that no useful or non-targeted bacteria presents in the environment will be harm, and therefore, reduce the possibilities of developing secondary infections (Goodridge and Abedon, 2003; Inal, 2003). However, on the other hand, the specificity of phage is also a disadvantage for phage therapy as phage will only attach a bacterium that matches to the specific strain. Thus, phage mixtures are often applied to improve the chances of success (Kumar et al., 2007).

1.5 Phages in Food and the Environment

A better understanding of the environment in which phages persist and their functions must be considered and gained before they can be used as biocontrol agents (Kadavy et al., 2000). Phages are ubiquitous, being found wherever bacteria are present (Biebricher and Gardiner,

1997; Marks and Sharp, 2000; Kennedy and Bitton, 1987). The concentrations of phages in soil have been calculated to be 10^7 pfu/g, while more than 10^6-10^7 pfu/ml and 10^8-10^{10} pfu/ml of phages are found in sea water and raw domestic sewage respectively, as estimated by electron microscopy (Sharp, 2001). With many reports detailing the isolation of phages from various foods, phages are suggested to be normal inhabitants of food ecosystems (Hudson et al., 2005). Furthermore, as phages could be very specific in infecting the bacteria, the pattern of infection by phages may be employed to distinguish bacterial species and strains, by a method called phage typing. Phage-typing is used in the diagnostic laboratory for the identification of pathogenic bacteria in assessing food quality (Hofstra et al., 1994; Väisänen et al., 1991).

Nevertheless, one way to employ phages as biocontrol agents is to apply them directly to processed foods, as many foods are distributed under refrigeration temperatures at which most foodborne pathogens do not divide (Hudson et al., 2005). However, other factors, such as the effect of the presence of non-targeted bacteria, the influence of physiochemical conditions (pH and water activity), thermo-tolerance of phage and bacteria, the emergence of phage-resistant mutants, relative numbers of phages, and effects of processing are needed to be taken into account when considering phage as food biocontrol agents (Hudson et al., 2005).

1.5.1 Foodborne Disease in New Zealand

Foodborne diseases are a widespread and growing public health problem globally. At year 2000, 2.1 million people was reported to died from diarrhoea diseases by World Health Organization, with most of the deaths caused by contamination of food and drinking water (World Health Organization, 2002). Furthermore, the epidemiological data from Europe, USA,

United Kingdom, Australia and New Zealand indicates that a considerable proportion of foodborne disease cases are caused by food prepared at home (Williamson et al, 1992; Meredith et al, 2001). As a support of this contention studies with New Zealand foodborne disease reported that approximately 119000 cases of foodborne disease were estimated per year (Lake et al., 2000; Scott et al., 2000), with days of lost production estimated to be approximately 497000 (Lake et al., 2000). The cost of these foodborne infectious diseases was estimated to be $55 million a year in lost productivity, medical and other associated cost (Scott et al., 2000). Thus, foodborne disease represents a major public health burden in the society. Although cooking will usually destroy pathogens in foods, disease may result if the food is undercooked, or are of ready-to-eat type (RTE) (Bennion and Scheule, 2004). Therefore, it is suggested that inactivation of pathogens with applications of phage might reduce foodborne disease incidence.

1.5.2 *Bacillus*

B. cereus has been recognized as a cause of foodborne disease since early this century with its role in foodborne disease established around 1950s (MacKenzie, 2004). *B. cereus* is a Gram positive, rod-shaped, spore-forming bacterium which can easily be isolated from foods (Nissen et al., 2002; Lake et al., 2004). *B. cereus* causes 1.2-4.5% of foodborne illnesses in New Zealand (Boxall and Ortega, 2003; ESR, 1998; Lopez et al., 2001, Naing et al., 1999; Perks et al., 2000; Thornley, 2002). Although illness caused by *B. cereus* is not a noticeable disease in New Zealand, it is thought that the foodborne disease incidence caused by pathogenic *B. cereus* is highly underreported due to its short duration and the difficulty of detecting the toxin from foods or isolates (Rhodehamel and Harmon, 1998; Lake et al., 2004).

B. cereus are ubiquitous in the environment (Lake et al., 2004) and causes two types of food poisoning, emetic and diarrhoea, both are caused by toxins. The emetic type is caused by a

highly stable toxin which survives at high temperatures or exposure to trypsin, pepsin and extreme pH (Lake et al., 2004). The emetic type of illness is typically caused by starchy products such as rice, dough, and potato. The diarrhoea type is characterised by abdominal pain and profuse watery diarrhoea. Recovery for both types of infection is rapid, usually within 12 to 24 h. Even though all people are susceptible to *B. cereus* infection, it is thought that for it to take effects, a high number of *B. cereus* is required to be taken (Lake et al., 2004). The infective dose of *B. cereus* ranges from 10^4 to 10^{11} cells per gram of food (Lake et al., 2004).

Most *B. cereus* strains are mesophilic with optimum growth temperature of 30-37°C, but a few strains are psychrotrophic, and can grow as low as 4-6°C (Lake et al., 2004). The minimum and maximum pH for its growth is 4.3 and 9.3 respectively. Growth of *B. cereus* is best in the presence of oxygen, and is inhibited in the presence of 0.1 % of acetic acid or 7.5 % of NaCl (Lake et al., 2004). The vegetative cells of *B. cereus* are readily killed by heat while the spores of *B. cereus* are easily spread by dust and are more resistant to heat and dehydration thus making *B. cereus* to be likely present in the environment and foods.

1.5.3 *Listeria*

L. monocytogenes has been known for at least 60 years but it has only been linked with foodborne disease since the early 1980s (Schuchat et al., 1991). *L. Monocytogenes* is recognized as an important food poisoning bacterium due to its ability to cause severe infections in both animals and humans (Schuchat et al., 1991). The primary habitat is soil and decaying vegetation but *L. monocytogenes* has also been suggested to be ubiquitous in the environments (Huss et al., 2004). Most isolates involved in human disease were found to be transmitted by food products and belong to three serotypes: 1/2a, 1/2b and 4b (Loessner, 1991; Loessner and Busse, 1990; Wendlinger et al., 1996; Pearson and Marth, 1990). *Listeria* mostly

affects people groups whose immune defence system is compromised, for example, elderly people, people with HIV infection, transplant patients and pregnant women (Huss et al., 2004). The incidence of listeriosis in New Zealand is rare (about 60-70 cases per year) and is comparable to that observed in other developed country (ESR, 2007). However, many attentions have been drawn onto this particular foodborne bacterium due to its high mortality rate (of approximately 20 – 40%) and the fact that FSANZ (Food Standards Australia New Zealand) suggested that only 33% of the incidents were reported. The outbreaks of listeriosis have been recorded due to the consumption of smoked mussels (Brett et al., 1998) and cooked meats (Sim et al., 2002). Furthermore, study done by Avery and co-workers suggested that the New Zealand isolates of *L. monocytogenes* are genetically diverse (Avery et al., 1996) which may draw more concerns on this bacterium.

L. monocytogenes is a Gram positive motile non-spore-forming intracellular pathogen. The optimum and minimum temperatures for *Listeria* growth are between 30-37°C and 0-2°C (Huss et al., 2004; ICMSF, 1996) respectively. The psychorotolerant property of *L. monocytogenes* allows it to grow well in refrigerated foods, while its halotolerant property makes it capable of growing and multiply in the present of up to 10% of NaCl (Huss et al., 2004; Seeliger, 1961). The incubation period of *L. monocytogenes* infection can be variable, ranging from one to ninety day (Huss et al., 2004). With such long period of incubation time, the identification of the source of pathogen can be difficult. *L. monocytogenes* infection is normally caused by foods that were contaminated by bacteria during processing or foods that are ready-to-eat (RTE). Highest contaminated incidences were reported to be found in meat, poultry, and seafood products (Farber and Peterkin, 1991).

1.6 Phages as a Biocontrol Agent of Foodborne Bacteria

1.6.1 The Potential of Phages as Biocontrol Agents

Phages are bacterial viruses that are also ubiquitous in the environment. It is suggested that for almost every bacterial species, at least one phage exists and can specifically infect and ultimately destroy a particular bacterial group (Walker, 2006). Given these characteristics, phages are thought to be valuable and promising alternatives method to traditional antimicrobials in the control of foodborne pathogens.

Phages could be used as biocontrol agents in pre-harvest applications, i.e. by treating food animals with phages prior to slaughter or by spreading on fruits or vegetables before harvesting to improve food safety (Randerson, 2003; Hudson et al., 2005). However, prolonged phages exposure to the bacteria is likely to result in reaching equilibrium between phage and host, due to mutation occurring in both phage and bacteria (Mizoguchi et al., 2003). Therefore, to avoid the selection for mutation and the arise of phage resistant of bacterial cells, introducing the phage to the raw products during the food processing stages might be a better strategy (Leverentz et al., 2001; Whichard et al., 2003).

Many foods are suggested to be contaminated with pathogens during food processing and harvesting. In addition, control of pathogens found on fruits and vegetables has become a major concern then on other type of foods. This is due to that fruits and vegetables are unlikely to undergo any further processing (i.e. cooking) to kill the pathogens that are present. Method of using phage as a biocontrol agent of pathogens is non-thermal intervention, which has the advantage especially on these foods. Furthermore, it has been demonstrated that phages successfully controls and significantly reduced the growth of *Campylobacter* and *Salmonella* on chicken skin (Goode et al., 2003), *Salmonella enteritidis* in cheese (Modi et al., 2001), and *L. monocytogenes* on meat and on fresh-cut fruit (Dykes and Moorhead, 2002;

Leverentz et al., 2003).

To be effective, phage must remain stable as infectious units in food. Phage stability is crucially affected by some physiochemical conditions such as pH, temperature, UV light, pressure, and chemical agents (i.e. Ethanol, isopropanol, NaOCl) that are generally applied to food processing environments (Hudson et al., 2005). Every phage might be stable in different physiochemical conditions. However, UV light, osmotic shock, and high pressure environment generally inactivates phages. Nevertheless, the characteristic of phage which allows it to survive in a wider temperature range and food processing environmental stresses than bacteria allow it to be a useful biocontrol agent (Marks and Sharp, 2000; Hudson et al., 2005; Goodridge & Abedon, 2003).

It is especially important to determine the phage's ability of controlling the bacterial cells in chilled food (4°C) because many foods are distributed under refrigeration, and most foodborne pathogens do not grow under this low temperature (Hudson et al., 2005). Other factors such as the timing of phage application, minimum host threshold to propagate enough phages to kill all target bacteria, host specificity of phages, and ability of the bacteria to avoid or develop resistance will also affect the success of phages as biocontrol agents in foods (Hudson et al., 2005).

1.6.2 Safety

Phages are ubiquitous in the environment which makes it inevitable to contact with these microorganisms. In support, Bergh and co-workers found that up to 2.5×10^8 plaque forming units (pfu)/ml of phage concentration was presented in natural unpolluted water (Bergh et al., 1989). However, consumers might still find the use of phages isolated from food more acceptable than others (Hudson et al., 2005). Research done by Ommnilytics has shown that

the majority of known phages do not have the ability to penetrate human tissues and cause disease, or damaging human cells or tissues even if phage did penetrated (Omnilytics, 2006). This was explained that although 19 families of virus that cause human disease are recognized by International Committee on Taxonomy of Viruses (ICTV), it is argued that the ICTV differentiating viruses in more research emphases manner rather than actual viral diversity (Omnilytics, 2006). Therefore, the virus that causes human disease does not necessary means bacteria phages. In support, the study of *E. coli* specific phages in human which are able to infect commensal and pathogenic *E. coli* strains , has failed to show any adverse effects in human (Bruttin and Brussow, 2005). In addition, an oral toxicity study with rats receiving high doses of *Listeria* phage Listex™ P-100 did not reveal any side effects (Carlton et al., 2005).

Plausible safety issues associated with phage and bacteria interactions such as killing of helpful bacteria, the release of toxins from lysed bacterial cells, and modification of specific bacteria to a higher virulence and broader phage's host range should be limited (Hudson et al., 2005; Goodridge and Abedon, 2003; Omnilytics, 2006). The hazards of killing helpful bacteria and releasing toxins could be avoided by employing phage that do not carry toxin genes and have a wider host ranges. It is suggested that due to the lost of the capsules, the bacteria resistance arising as a result of phage application are generally less virulent than the response of bacteria with antibiotics (Hudson et al., 2005). Temperate phages should be avoided to be used as a biocontrol agent in food to avoid the possibility of antagonistic co-evolution that increases host resistant and broader phage's host range (Hudson et al., 2005; Goodridge and Abedon, 2003). So far, study suggested that phage therapy has indicated a history of safe use with few adverse effects in animals and humans (Hudson et al., 2005). However, more studies are required for the complex phage-bacterial interactions.

1.7 Previous Works on Phage Isolation

1.7.1 Isolation of *Bacillus cereus* Phages

Although many *Bacillus* phages were isolated and studied in the literature (Ackermann et al., 1978; Romig et al., 1961; Strauch et al., 1989), to my knowledge, not as many *B. cereus* phages were studied in comparisons to *L. monocytogenes* phages. This may result from low number of reported incident and non-severer infection of *B. cereus*, therefore drawing lesser attention onto this particular bacterium. The isolation of *B. cereus* phages was firstly achieved using Adams' soft agar method from soil samples by Thorne (1968). The phage isolated, namely CP-51, was propagated on *B. cereus* NRRL 569 and was later demonstrated to be capable of generalized transduction. CP-51 is also active on several other strains of *B. cereus* (6464, 9239 and T) and even *B. anthracis*, but is inactive on *B. subtilis* and *B. licheniformis* (Thorne, 1968; Yelton and Thorne, 1970). The CP-51 was suggested to be a temperate phage due to the present of a small colony that is often seen to be emerging in the centre of CP-51 plaques, suggesting it to be a temperate phage. However, spontaneous mutants of CP-51 were observed to produce completely clear plaques (Thorne, 1968). The unique characteristic of CP-51's towards cold temperature makes it very unstable. In 24 h, 90% of the initial phage concentration was lost when stored at 2 to 4°C (Thorne, 1968). The optimal temperature of plaque formation was 15°C for CP-51 (Thorne and Holt, 1974), which is outside the optimum growth temperatures of *B. cereus* (i.e. 30-37°C) (Lake et al., 2004). Electron microscopy images showed CP-51 belongs to *Myoviridae* family of phages, with a head size of 89.7 nm and a contractile tail of 20.0 x 159.9 nm (Yelton and Thorne, 1971).

During their study, Yelton and Thorne also found phage CP-53, in lysates of CP-51, propagated on *B. cereus* strain 6464 which could transduce auxotrophic mutants of strain 569 to prototrophy (Yelton and Thorne, 1971; Yelton and Thorne, 1970). Phage CP-53 is morphologically distinct to CP-51 and is the same phage that Altenbern and Stull isolated

(Alterbern and Stull, 1964). CP-53 phage gives very turbid plaques and is shown to be a smaller phage with head size of 66.2 nm and a long and non-contractile tail size of 12.5 x 275.9 nm (Yelton and Thorne, 1971).

Other *B. cereus* phages such as Bace-11 and Bastille were also reported in the literature (Ackermann et al., 1995; Eiserling and Boy de la Tour, 1965). However, not many followed on their characteristic studies and their potential as a biocontrol agent in food. Hence, these studies available in literature will provide a good guideline on the method of isolation and comparison on the morphology of phages isolated for this study. Furthermore, this study will also evaluate the potential of *B. cereus* phages isolates as a biocontrol agent in food.

1.7.2 Isolation of *Listeria* phages

Listeria phages were first reported in 1945 (Schultz, 1945) and since then, at least 400 phages of *Listeria* species have been isolated from the environment (Estela et al., 1992; Loessner, 1991; Loessner and Busse, 1990; Ortel and Ackermann, 1985; Waldor et al., 2005). It is suggested that the isolation of *Listeria* phages is relatively easy (Zink and Loessner, 1992). Standard double-layer agar plates made with standard media, such as tryptose or brain heart infusion (BHI) medium are sufficient to allow *Listeria* phage propagation with its bacterial host (Waldor et al., 2005). Most of *Listeria* phages reported in the literature belong to the *Siphoviridae* family (characterised by having isometric capsids and a long noncontractile tail) (Ackermann et al., 1981; Rocourt et al., 1982) with few belonging to the *Myoviridae* family (characterised by isometric capsids and long tail with contractile sheath) (Bradley, 1967; Rocourt et al., 1982). In addition, most phages isolated are temperate phages which only recognize individual serotype groups of host bacteria. Only few virulent *Listeria* phages (e.g. A511) can attach to strains from all *Listeria* species and serotypes (Zink and Loessner, 1992).

All *Listeria* phages isolated so far have double-stranded DNA genomes, with genome size ranging from 36 to 130 kb pair, and G+C contents of 34.7 to 40.8 mol % (Waldor et al., 2005). A511 is a particularly interesting virulent phage because it has a broad host range. It produces relatively large plaques on lawn of double-layer agar, and the inner core of plaques is surrounded by a zone of secondary lysis due to the diffusion of the phage endolysin liberated from the lysed cells (Waldor et al., 2005). A511 belong to *Myoviridae* family and has a nonflexible, contractile tail about 180 x 20 nm in length and width respectively and an isometric head of 88 nm and a base plate of 20 x 40 nm in height and width respectively (Zink and Loessner, 1992; Waldor et al., 2005). A511 has a large genome size of 134.5 kb base and an unusual wide host range that lyses approximately 95% of *L. monocytogenes* strains of serotype 1/2 and 4 while remaining strictly specific for members of the genus *Listeria* (Waldor et al., 2005). Due to the well studied characteristics of A511 phage, it is used as reference phage to ensure the methodology in this study is efficient.

Listeria phages are good and useful for their applications in phage typing of *L. monocytogenes* strains (Radding, 1971; Marquet-Van Der Mee and Audurier, 1995). Furthermore, the biological specificity of *Listeria* phage allows the application to detect and control host bacteria, primarily with respect to foods. Studies of treatment with *Listeria* phage have been done on honeydew melon and apple (Leverentz et al., 2003; Leverentz et al., 2004). Most effective way of controlling *Listeria* bacterium was observed when applied phage with a concentration of 10^8 pfu/ml and up to 1 h after processing of the melons (Leverentz et al., 2004). Although phages could be isolated from low acid environments (Leverentz et al., 2004), however the growth of the *L. monocytogenes* hosts far outweighed the numbers of phages that survived (Leverentz et al., 2004) in apples (approximately pH4), which shows the limitation of phage treatment at the moment.

Furthermore, there are two commercial *Listeria* phage products, Listex™ P-100 and LMP-102™ which are used in protection of plant- and animal-derived food products (Liu et al., 2004; Petty et al., 2007; Skurnik and Strauch, 2006). Phage P-100 was originally isolated from a wastewater sample taken from a dairy plant in Germany in 1997 (Carlton et al., 2005). It is a phage preparation derived from phage P-100 that targets *L. monocytogenes* along with few other species of *Listeria* in foods. EBI Food Safety (EBI Food Safety, 2006b) claims 100% effectiveness of performance when Listex™ P-100 was applied with the host strain. Another product, LMP-102™, is a phage preparation produced by Intralytix that also targets *L. monocytognenes*. LMP-102™ consists of six bacteriaphages that are found naturally in the environment and is currently targeted for use on RTE products and on food processing equipments. It is claimed that food applied with LMP-102™ does not contain any preservatives or allergens, and is shown to be effective in reducing the bacterial load by 100-1000 fold (Intralytix, 2006c).

1.8 Research Objectives

This thesis explores the potential to use phages for biocontrol of the foodborne pathogens, namely *B. cereus* and *L. monocytogenes*. *Bacillus* phages isolated in this study were tested and characterised in various ways for a better understanding of host and phage interaction. *Bacillus* phages were added to mashed potatoes at different temperatures to ensure their effect as biocontrol agent. Different methods for isolation of virulent *Listeria* phages from the environment were applied, and success in isolation was achieved in the end. Isolated phages were obtained late in these studies, but the characterisations completed are described. The findings of this thesis will add to the current knowledge of phages and may draw us closer to determining if phages can be used to as biocontrol agent on food.

1.9 Hypotheses

- Phages have the potential to be use as a biocontrol agent in foods.
- Phages and their hosts are present in food and can be isolated from foods.
- Phage activities against *Bacillus* and *Listeria* in laboratory medium can be predictive of their behaviour in food.

Chapter 2

Materials and Methods

2.1 *Bacillus* Culturing

Bicillus cereus was grown in nonselective Brain Heart Infusion (BHI) (Dufrenne et al., 1995; Beuchat et al., 1997) medium (Appendix A.2) unless otherwise specified in results. BHI was also used for the Agar Layer Method (Adams, 1959) for making *Bacillus* titres. The *Bacilli* are cultured in BHI broth (Appendix A.2.1) until they reach the exponential phase of growth, usually within 3-4 h. The BHI overlays and BHI agar were prepared for *B. cereus* testing. (Appendix A.2.2 to A.2.6). All BHI plates and broth were incubated in 37°C unless indicated otherwise.

2.2 *Listeria* Media and Growth Conditions

Tryptic Soy Broth (TSB) (Appendix A.3.1) (Petran, et al., 1993; Kwang et al., 1994) was used to prepare bacteria suspensions by overnight (18-22 h, unless stated otherwise) incubation at 30°C. TSB plates and overlays (Appendix A.3.2 to A.3.4) were used for the Agar Layer Method to visualise plaques. Isolated samples were grown in Fraser broth with supplements (Appendix A.3.5 and A.3.6) (Fraser and Sperber, 1998) as trials to promote the visualise plaques of *Listeria* phages.

2.3 Reference Cultures

2.3.1 Two *B. cereus* cultures were isolated from six packs of Cinderella instant mashed potatoes, by 1:10 dilution of the instant potatoes in SM buffer. Bacteria isolated from the potatoes were purified by plating on the Mannitol yolk polymyxin (MYP) (Mossel et al., 1967)

(Appendix A.2.7) selective agar. The isolates were then confirmed by rapid confirmatory test (staining test) (Holbrook and Anderson, 1980), MYP test (Mossel et al., 1967; Donovan, 1958; Coliner, 1948), heamolysis test and microscopy for crystal formation (A. Hudson, personal communication, 2007) to distinguish them from species that are closely related to *B. cereus* (i.e. *B. thuringiensis*, *B. subtilis*, *B. mycoides* and *B. anthracis*). One strain of *B. thuringiensis* subsp. *israelensis* (NZRM 2981) and one strain of *B. cereus* (NZRM5) which was obtained from the New Zealand Reference Culture Collection, Environmental Science and Research (ESR), Christchurch Science Centre, were used for isolation of phages. *B. cereus* (NCTC 11143 & NCTC 11145) obtained from ERS, New Zealand Reference Culture Collection, Christchurch Science Centre with *B. thuringiensis* (DSM 2046), *B. licheniformis* (DSM 603), *B. subtilis* (NTCT 3610), *B. mycoides* (ATCC 6462) and *B. megaterium* (NCTC 10343) were obtained from Fonterra, Palmerston North, New Zealand (S. Flint, personal communication, 2007). One of the *B. mycoides* strain isolated from raw mussel was provided by TeckLok Wong, ESR (T. Wong, personal communication, 2007), and was used in addition for host range determination.

2.3.2 *Listeria monocytogenes* (LM) strains 3009 and 2000/47 were provided by Fonterra (Palmerston North, New Zealand) and used for isolation of *Listeria* phage from the environment, along with *Listeria*-specific phage, A511 (Zink and Loessrov, 1992; Wendlinger et al., 1996), used for reference phage control. A511 phage was chosen because strain LM 3009 is in its host range and because it is so well characterized, it served as a control. However, A511 phage was tested and shown a negative result with LM strain 2000/49, which was the strain associated with foodborne outbreaks. *L. inoocua*, *L. ivanovii*, *L. weishimeri*, *L. grayi*, *L. monocytogenes* (NZRM 44, 2592, 2594, 3370, 3449, 3450,) were obtained from New Zealand Reference Culture Collection, ESR, Christchurch Science Centre, and used in

addition of above two LM strains as indicator hosts for host range determination of *Listeria* phages.

2.4 Agar Layer Method (Overlay Method)

Agar base plates were made using 3-4 ml of BHI or TSB media, augmented with 1% agar (Adams, 1959) for *Bacillus* and *Listeria* testing respectively. The soft-agar overlays required 0.6 % (w/v) (Adams, 1959) of agar and 0.4 % (w/v) of agarose (Appendix A.2.5 and A.3.4) were stored in 4 ml quantities for *Bacillus* and *Listeria* respectively. Soft-agar overlay tubes were melted by autoclave steaming at 100 °C for 15min or by boiling in a waterbath when required, and kept in a waterbath of 48°C before use.

Bacillus in the exponential phase of growth was mixed with phage and the BHI broth was brought to 1.8 mM of $MgSO_4$, 0.8 mM of $CaCl_2$ (Cooney, et al., 1975) (Appendix A.1.2 and A.1.3) in each overlay before pouring onto base plates. *Listeria* tests were preformed the same way except that the broth was brought up to 1.25 mM of $CaCl_2$ (Loessner and Busse, 1990). The soft-agar was swirled for a consistent covering and left to dry at room temperature (i.e. 22°C) for 15-30 min before inverting and incubating at desired temperature overnight.

2.4.1 Preparing a *Bacillus* Lawn in a Soft-Agar Overlay

To isolate *Bacillus*-specific phages from the samples described above, the bacteria were streaked on MYP selective plates and incubated at 37°C overnight to obtain isolated colonies. Bacterial suspensions of indictor host were then prepared by transferring one isolated colony into 20 ml of BHI broth and incubating at 37°C overnight. 15 ml of fresh BHI was inoculated with 0.1 ml of the 18-22 h old bacteria suspension and incubated with reciprocation shaking (100 rpm) at 37°C for 3-4 h. To create a bacterial lawn sufficient for visualizing plaques, 0.1

ml of the 3-4 h culture suspension was mixed into 4 ml of soft-agar tube and overlaid on a BHI base plate.

2.4.2 Preparing a *Listeria* Lawns in a Soft-Agar Overlay.

Since *Listeria* does not sporulate like *B. cereus*, a pure colony of *Listeria* was prepared by streaking on PALCAM selective plate (Van Netten et al., 1989) (Appendix A.3.7) and incubated at 30°C overnight. A single *Listeria* colony was inoculated in 20 ml of TSB broth and incubated overnight with reciprocation shaking (100 rpm) at 30°C. 0.1 ml of the overnight bacteria suspension was mixed into 4 ml of soft-agar tube and overlaid on TSB base plates to create a lawn dense enough to visualize plaques.

2.5 Isolation of *Bacillus*-Specific Phages

Several soil, raw milk, rice and spice samples were collected from various locations within Christchurch. Reference cultures were used as indicator hosts, with samples prepared in various methods for phage isolation.

2.5.1 Preparing Samples for Serwer's Direct *Bacillus* Phage Isolation (Serwer et al., 2004)

A 1:10 dilution of raw milk was made with SM buffer (Appendix A.1.1), while others 1:10 dilution of soil, rice and spice samples were made with SM buffer respectively in sterilize bags with an inner filter and left at room temperature for 10 min before mixing for 3 min. 0.1 ml of these dilutions were used to test for the presence of phages.

2.5.2 Preparing Samples for Direct *Bacillus* Phage Isolation

Raw milk samples were prepared by centrifuged at 3000 x g for 10 min and 1ml of supernatant was carefully transferred into a new centrifuge tube without disrupting the top fat layer of the raw milk. The supernatant of raw milk was then diluted by 1:10 with SM buffer and filter-sterilized (0.22 µm in pore size, Millex GP 33mm) supernatant was tested for the presence of phages by plating on four different *Bacillus* strains.

For other samples, a 1:10 dilution of soil, rice and spice were made with SM buffer respectively in a sterilize bag with inner filter and left at room temperature for 10 min before mixing for 3 min. Samples were then transferred through filter to a sterilized centrifuge tube (50 ml) and centrifuged at 3000 x g for 10 min. Supernatants were filter-sterilized (0.22 µm in pore size) and tested for the presence of phages by plating on four different *Bacillus* strains.

2.5.3 Preparing Enrichment of Samples for *Bacillus* Phage Isolation

All samples were diluted by 1 in 10 with SM buffer and enriched by addition of 100 µl of exponential phage indictor hosts. After incubation at 37°C overnight, the enrichment was centrifuged and filtrated, as described above.

For milk samples, 1 ml of bacteria suspensions of indictor host were added into 10 ml of milk sample and incubated at 37°C overnight. The enrichment was centrifuged at 3000 x g for 10 min after incubation. 1 ml of the supernatant was carefully transferred into new centrifuge tube without disrupting the top fat layer from milk samples. Samples were then diluted and filtrated as described in direct *Bacillus* phage isolation method.

2.5.4 Preparing Enrichment of Samples with Addition of Beef Extract for *Bacillus* Phage Isolation

All samples, including milk samples, were diluted by 1:10 with beef-extract (3%, Bacto®, pH = 7.5) (Appendix A.1.6) (Seeley and Primrose, 1982) and mixed for 30 min on rotary shaker (40 rpm) at room temperature. Samples were then enriched by addition of bacteria suspensions of indicator host (0.1 ml), followed by centrifugation and filtration of the supernatant as described above.

2.5.5 Assaying Samples for *Bacillus* Phages

For directed isolation method, the soft agar overlay (Adams, 1959) consisted of the exponentially growing bacterial culture (100 µl), filtered supernatant (100 µl), 1.8 mM of $MgSO_4$, 8 mM of $CaCl_2$ (Cooney, et al., 1975) and molten soft agar (1, 0.8, 0.6 and 0.4 % (w/v) agar in BHI broth, Appendix A.2.3 to A.2.6) at 48°C. The mixture was poured into pre-solidified BHI plates and incubated at 37, 24 and 10°C for 24, 48 and 96 h, respectively.

For enriched samples, soft agar consisted with above mixture in 0.8 % (w/v) of molten agar (in BHI broth) at 48°C was poured into pre-solidified BHI plates and incubated overnight at 37°C.

2.6 Isolation of *Listeria*-Specific Phages

Animal sewage, animal faeces, Effluent, and compose samples were collected from Tegel Foods Ltd, (Hornby, Christchurch), PPCS Ltd, (Belfast, Christchurch) and households of ESR staff. Reference cultures LM 2000/47 and LM 3009 were used as indicator hosts, with samples prepared by methods listed below for phage isolation.

2.6.1 Preparing Samples for Serwer's Direct *Listeria* Phage Isolation (Serwer et al., 2004)

A 1:10 dilution of animal sewage and effluent samples were made with SM buffer, while a 1 g sample of dog and animal faeces was vortex missed in 9 ml of SM buffer to make a 1:10 dilution. All the 1:10 samples were mixed and left on rotary shaker (40 rpm) at room temperature for 30 min before testing for present of *Listeria* phage.

2.6.2 Preparing Samples for Direct *Listeria* Phage Isolation

Samples were diluted and mixed as described above before pellet formed by centrifugation at 3000 x g for 10 min and filtered through a 0.22 µm filter.

2.6.3 Preparing Samples for Direct *Listeria* Phage Isolation with Addition of Beef Extract

Samples were prepared by 1:10 with beef extract (3%) (Seeley and Primrose, 1982) and mixed on rotary shaker at room temperature for 30 min before centrifuging at 3000 x g for 10 min and filtering through a 0.22µm filter.

2.6.4 Preparing Enrichment of Samples for *Listeria* Phage Isolation

A 1:10 dilutions of pre-enriched samples were prepared as described above in section 2.6.2, with 0.1 ml of bacteria suspensions of *Listeria* prepared as described in section 2.4.2 were added into each sample and incubated at 30 °C overnight. The supernatant of enrichment was centrifuged and filtered, as described above.

2.6.5 Preparing Enrichment of Samples with Addition of Beef Extract for *Listeria* Phage Isolation

A 1:10 dilution of pre-enriched samples were made with beef extract (3%) (Seeley and Primrose, 1982) and mixed using a rotary shaker at room temperature for 30 min. Bacteria suspensions of *Listeria* hosts were prepared and 0.1 ml was added into each dilution and incubated at 30°C overnight. Enrichment was then centrifuged and filtered as described above.

2.6.6 Preparing Samples With Fraser Broth (Fraser & Sperber, 1988)

All samples prepared in methods described above were re-prepared with 1:10 dilution in Fraser broth instead of SM or 3% of beef extract. Centrifugation at 3000 x g for 10 min and filtration through a 0.22µm filter were carried out in each method.

2.6.7 Assaying Samples for Isolation of *Listeria* Phages

For Serwer's method (Serwer et al., 2004), 100µl of samples were transferred onto the TSB base plate. Followed with mixing and pouring the mixture of 100µl of bacteria suspension added into the overlay which containing 0.5 % (w/v) of agar and 1.25 mM $CaCl_2$ (Loessner and Busse, 1990) onto TSB base plates. Plates were swirled immediately for consistent coverage. Plates were incubated at 30°C overnight before screening for plaques.

Initially, the soft agar overlay consisting of 0.5% (w/v) agar in TSB broth was molten and kept in 48°C waterbath. 100µl of exponentially bacterial culture and samples prepared were added into the overlay which containing 1.25 mM $CaCl_2$ (Loessner and Busse, 1990). The mixture was vortex gently and poured onto pre-solidified TSB plates and incubated at 30°C overnight before screening for plaques.

For samples prepared by diluting with Fraser broth (Serwer et al., 2004), 100µl of bacteria suspension and samples prepared were added into the molten overlay (0.5% (w/v) agar in TSB broth) that contained 1.25 mM $CaCl_2$ and 0.01% (w/v) of Fraser supplement (Serwer et al., 2004). Overlay tubes were mixed and pour onto TSB plates and incubated at 30°C overnight.

2.6.7.1 Immunomagnetic Separation (IMS) (Favrin et al., 2003; C. Billington, personal communication, 2007; Wright et al., 1994)

2.6.7.1.1 Washing Anti-*Listeria* Dynabeads

Anti-*Listeria* Dynabeads (Dynabeads®) are made of uniform, paramagnetic, polystyrene beads with purified anti-*Listeria* antibodies bound covalently onto the surface. The Dynabeands are designed for a rapid isolation and concentration of *Listeria* directly from pre-enriched samples. The antibodies coated onto Dynabeads will specifically bind *Listeria* and form a complex which were subsequently separated and isolated from the sample matrix using a magnetic particle concentrator, Dynal MPC®-S.

The Dynabeands were resuspended by gently mixing the vial for 60 sec before 1 ml of beads were removed to an eppendorf tube. The tube was placed in magnetic holder and shaken for 2 min to separate the beads. The liquids were carefully removed by pipette and 1 ml of PBS/BSA (Appendix A.4.3) was added into the tube and mixed for concentrating the beads. Another washing step was taken and beads were resuspended in 1 ml of PBS/BSA mixture and stored in 4°C and mixed before use.

2.6.7.1.2 Testing of Dynabeads

Bacteria suspension of LM 2000/47 was prepared as described in Section 2.4.2., and was serially diluted by ten fold for six times (i.e. 10^8, 10^7, 10^6, 10^5, 10^4 10^3 and 10^2 colony forming unit (cfu)/ml) with SM buffer and 100µl was transferred and spread on TSB plates for colony counts. 1 ml of each bacteria suspension dilutions were transferred into enpperdorf tube with 50µl of Dynabeads added to it. Tubes were placed and rotated for 10 min on the magnetic holder. Supernatant were removed carefully by pipette and Dynabeads were resuspended in 1 ml of SM buffer followed by 3 min of rotation on the magnetic holder. This washing step was repeated 3 times and Dynabeads were finally resuspended in 0.1 ml of SM buffer. To ensure the Dynabeads' activity, 50µl of this above resuspended mixture from each dilution of bacteria suspension were transferred and spread onto TSB plates in triplicate. All plates were incubated in 30°C overnight before the host recovery ratio of Dynabeads were investigated.

2.6.7.1.3 Isolating *Listearia* Phage Using IMS

Bacteria suspensions of LM 2000/47 and LM 3009 were prepared and 10 ml of each indicator host was transferred and mixed together in a sterile tube. The rest of the bacteria suspensions were kept in ice for later use. 9 ml of each sample prepared from section 2.6.1 to 2.6.6 were placed on the magnetic holder with 1 ml of host mixture added to them. Tubes were left on rotary shaker (40 rpm) at room temperature for 20 min before 250 µl of Dynabeads were added to them and rotated for 10 min. Tubes were then placed on the magnetic holder and rotated for 3 min for IMS. Supernatant of these tubes were removed by pipette before 10 ml of SM buffer was added back for resuspension and then returned to the magnetic holder for 3 min for IMS. Washing with SM buffer was repeated. After the washing procedure, beads were resuspended in 0.5 ml SM buffer. Overlays were carried out by adding 0.1 ml of this SM

buffer mixture and host suspension to molten TSB soft-agar that containing 1.25 mM $CaCl_2$. Plates were incubated overnight at 30°C.

2.6.7.2 0.4 % Agarose Concentration With Incubation Temperature of 22°C

An overlay was carried out again with all the samples prepared from Sections 2.6.1 to 2.6.6 with two alterations on the method 0.4% (w/v) of agarose instead of 0.6% (w/v) agar in overlay tubes were prepared and plates were incubated at 22°C instead of 30°C for plaque visualization.

2.7 Propagating Plaques and Purifying Phages

Plaques observed from overlay method were stabbed with a 0.1 ml wide-bore pipette tip through to the base agar and a plug of agar removed. The plug was dispensed into tin-foil wrapped sterile 3 ml bijou bottle containing 2 ml of SM buffer and left on the rotary shaker (40 rpm) at room temperature overnight to allow the phages to be diffused from the agar to the SM buffer. After the diffusion, samples were gently mixed and the liquid was filtered through a 0.22 µm pore-size filter and stored at 4℃ in a tin-foil wrapped sterile 3 ml bijou. The filtrate of the propagated plaque was then serially diluted by ten fold in SM buffer and an overlay was inoculated with fresh indicator host (0.1 ml) and dilution of filtrated plaque (0.1 ml) with 1.8 mM $MgSO_4$ and 0.8 mM $CaCl_2$ for *Bacilius* phage and 1.25 mM $CaCl_2$ for *Listeria* phage to produce more plaques. If plaques were observed from the overlay plate, this procedure was then repeated at least three times until plaque morphologies were consistent.

2.8 Preparing High Titre Stocks of Purified Phages

Once purification of phages was achieved by method described in Section 2.8, concentrated stocks of the phage were required for storage and further characterization studies. The filtrate

of the purified phages was serially diluted by ten fold in SM buffer to give a concentration that would provide confluent lysis of the host in a soft-agar overlay plate. The dilution giving the most confluent lysis was chosen, and 20 plates were overlaid for each phage stock. After overnight incubation at desire temperature, 5 ml of SM buffer was added to each plate and left at room temperature for 4-6 h, on rotary shaker at 40 rpm.

The 5ml of SM buffer was then decanted into centrifuge tubes and centrifuged at 3,000 x g for 10 min, while the soft-overlay layer was scraped from the base plate into a sterile bag wither inner filter containing 4 ml of SM buffer per each plate. The contents were mixed in a stomach bag for 3 min and then left at room temperature for 20 min to allow the phage to disperse from the overlay. The liquid was then decanted into centrifuge tubes through the filter and centrifuged at 3,000 x g for 10 min which was sufficient for separating the overlay agar. The supernatant was collected from both tubes and filtered (0.22 µm), followed with overnight centrifugation at 26,916 x g at 4°C.

The supernatant was then discarded and 2 ml of SM buffer was added to each tube. The tubes were tin-foil wrapped and left on the rotary shaker (40 rpm) for 4-6 h at room temperature allowing the very small pellets from centrifugation to be dissolved and filtered (0.22 µm). The titire of the phage stock was then enumerated by the overlay method with appropriate indicator host strain.

2.9 Preparation for Transmission Electron Microscopy (TEM)

2.9.1 Making Fresh High Titre Stocks

Phage stocks were plated using the overlay method to give confluent lysis over 20 plates for each sample. After overnight incubation, 5 ml of SM was added to each plate and left at room

temperature for 4-6 h with constant swirling by rotary shaker (40 rpm). The liquid then was decanted into tubes and centrifuged at 3,000 x g for 10 min and then the supernatant was filtered (0.22 µm). The filtrate was then centrifugated at 26,916 x g overnight at 4°C. The supernatant was carefully discarded next day, and the clear pellet remaining was gently resuspended with 1 ml of 0.1 ammonium acetate and centrifugated at 26,916 x g overnight at 4°C again. This washing step was then repeated twice to give a total of three washes. After the final wash, supernatant was gently discarded and the pellet was resuspended in 1 ml of 0.1 M ammonium acetate (Appendix A.5.1) and then filtered (0.22 µm) and stored at 4°C. The target titre of the stocks was $>10^{10}$ pfu/ml to allow visualization by electron microscopy and the phage titre stocks was immediately packed into travel container with ice-pack and courier to Otago Centre for Electron Microscopy (OCEM).

2.9.2 Mounting Phages for Visualization with a TEM (Done by OCEM)

Using a transmission electron microscope (TEM), the morphologies of phages were examined by a single negative staining method. 10µl of phage stock was applied to a carbon-coated 300 mesh Copper grid (Appendix A.5.3) and left for 1 min at which time the excess liquid was blotted off with filter paper and the grid given time to air dry. Negative stain of 1 % phophotungstic acid (PTA, aqueous, pH 6.5) (Appendix A.5.2), was applied and blotted off immediately with filter paper and the grid air dried. The grid was then loaded into the microscope and all TEM images were taken at the same TEM magnification of 135,000 x magnification using the CM100 TEM, 100 kV accelerating voltage and with the specimen at the eucentric point. At least 20 images were taken of each phage sample. Images were supplied with a scale bar for magnification checking purposes. This can be calibrated against the known size of the catalase crystals by measuring 10 catalase crystal distances and multiplying the number by the 8.75 nm, which is the distance between crystal lattices. There

was a ‘pre’ and ‘post’ session of catalase micrograph taken to indicate any magnification error change which may have occurred during the session (R, Easingwood, personal communication, 2008).

2.10 Host Range Determination

High titre stocks were serially diluted by ten fold by mixing 0.1 ml of stock in 0.9 ml of SM buffer. Bacteria suspensions of the original and possible indicator host strains were prepared as described in section 2.4.1 and 2.4.2

Using the spotting technique, each overlay was inoculated with 0.1 ml of bacteria suspension, and poured on a base plate previously labelled as a grid-like format for identification of each stock dilution. Once the overlay was dry, 10 µl of phage stocks corresponding to final titres of 10^3, 10^5, 10^7 pfu/ml, were positioned across the overlay. The plates were left in a safety cabinet at room temperature for 30 min until the spots were dry and then incubated overnight at desire temperature. Plates were examined for plaques after overnight incubation. Inhibition of the bacteria to form clear zones in a lawn was investigated as possible phage activity while lysis from without could also be observed from clear zones at high concentrations of phages.

2. 11 Single-Step Growth Experiment

2.11.1 Growth Curve of *B. cereus*

Initially, 50 ml of BHI broth was inoculated with a fresh *Bacillus* colony from an MYP plate and, incubated overnight at 37°C in shaking waterbath (100 rpm) along with two sterile glass bottles (250 ml) containing 45 ml of BHI broth. On the following day, 5 ml of the bacteria suspension was transferred into both glass bottles, incubated at 37°C shaking waterbath (100 rpm). The OD_{660} reading was taken every 30 min until it reached >0.8, which has been

assumed to be representing 10^8 cfu/ml of culture. At every reading time, samples were also collected and serially diluted by ten fold in BHI broth. 0.1 ml of each diluted sample was spread on a BHI base plate and incubated overnight at 37°C. This step was performed in triplicate. From this particular set of experiments, the relationship between OD reading and the colony count could be determined, which was used as an indicator of number of bacteria presented in the broth for later experiments.

However, by the method described above, it does not give a linear relationship between OD reading and the colony count. This was suspected to be due to the interfering of *Bacillus* spores on the OD reading. Therefore, the method was altered slightly by working backward from OD reading to colony count to find the linear relationship between them.

Bacteria suspension was prepared and 5 ml of it was transferred into 20 ml of fresh BHI broth as described above. The freshly inoculated BHI broth was incubated at 37°C for 3-4 h until its OD reading reached 0.8 at 660 nm and was kept on ice. 0.1 ml of the bacteria suspension with OD reading of 0.8 was plated in triplicate on BHI plates in ten fold serial dilutions with BHI broth. The bacteria suspension was then diluted by a factor of two for four times with BHI broth until OD dropped to 0.02 at 660 nm. For each dilution, OD reading was taken and 0.1 ml of each bacteria suspension dilutions were transferred and spread on BHI plate and incubated overnight at 37°C. The colony counts, of each bacteria suspension dilutions were recorded on the next day.

2.11.2 Lysis Ability of *Bacillus* phage

Bacteria suspension was prepared with 5 ml of bacteria suspension transferred into 20 ml of fresh BHI broth and incubation at 37°C for 3-4 h (this method was used for all *Bacillus*

testing later on, unless stated otherwise). Three glass tubes (i.e. tube A, B, and C) containing BHI broth with 1.8 mM $MgSO_4$ and 0.8 mM $CaCl_2$ were prepared and kept at 37°C. Phage titre stocks were diluted with SM buffer and transferred to tube A and B to give a final concentration of 10^6 and 10^5 pfu/ml. When the culture had achieved an OD reading of approximately 0.8, at 660 nm, the bacteria suspension was transferred to all three tubes to give a final concentration of 10^7 cfu/ml. The remaining portion of the bacterial suspension (with OD reading of approximately 0.8) was kept on ice for quantifying the number of host cells present at the start of infection and used as the indicator culture for the plaque assay. The remaining phage were stored on ice until assayed.

The OD reading was taken in 30 min interval until the OD reached its maximum (i.e. > 0.8) at 660 nm. At every reading time, sample was serially diluted by a factor of ten, and were transferred and spread on BHI plate as described above. In addition, at zero and the final reading time, culture was collected and serially diluted by a factor of ten with SM buffer. An overlay was carried out for each culture dilution for plaque visualization. All plates were incubated overnight in 37°C. Colonies and plaques were counted in relationship of OD readings on the following day.

2.11.3 Lysis Ability of *Listeria* phage

Procedures for testing lysis effect of *Bacillus* phage were repeated with alterations of TSB medium, incubation temperature of 22°C, and $CaCl_2$ concentration (1.25 mM).

2.11.4 Single-Step Growth Curve of *Bacillus* Phage

BHI broth was equilibrated to the set temperature for at least 30 min prior to starting the experiment. Bacteria suspension of *Bacillus* was prepared with the double dilution technique

described above. Phage and bacteria suspension was diluted with SM buffer and BHI broth respectively and was transferred to a glass tube of BHI broth which contained 1.8 mM $MgSO_4$ and 0.8 mM $CaCl_2$. The final concentrations of phage and bacteria suspension in the tube were 10^6 pfu/ml and 10^7cfu/ml respectively. The remaining portion of the bacterial suspension was kept on ice for quantifying the number of cells present at the start of infection and to b used as the indicator culture for the plaque assay. The remaining volume of the phage dilution was stored on ice until assayed.

To give a count of unadsorbed phages, a 1:10 dilution was made with BHI broth and centrifuged at 3000 x g for 10 min to separate the unadsorbed phages from bacteria, after phage infection (i.e. 5 min after infection). The supernatant was filtered and the titre of unadsorbed phages was found by overlays. The glass tube was also centrifuged at 3000 x g for 15 min to separate the phage and bacteria culture. After centrifugation, the supernatant was discarded, and pellet was resuspended in 10 ml of BHI broth and was diluted with BHI broth to final concentrations of 10^4, 10^5, 10^6 and 10^7cfu/ml. A set of overlays was made using material from time zero in 10 min interval for 140 min.

The quantification of cells in the culture was achieved with serial dilution by a factor of ten of the suspension with BHI broth. All plates were incubated overnight at 37°C before host colonies were counted.

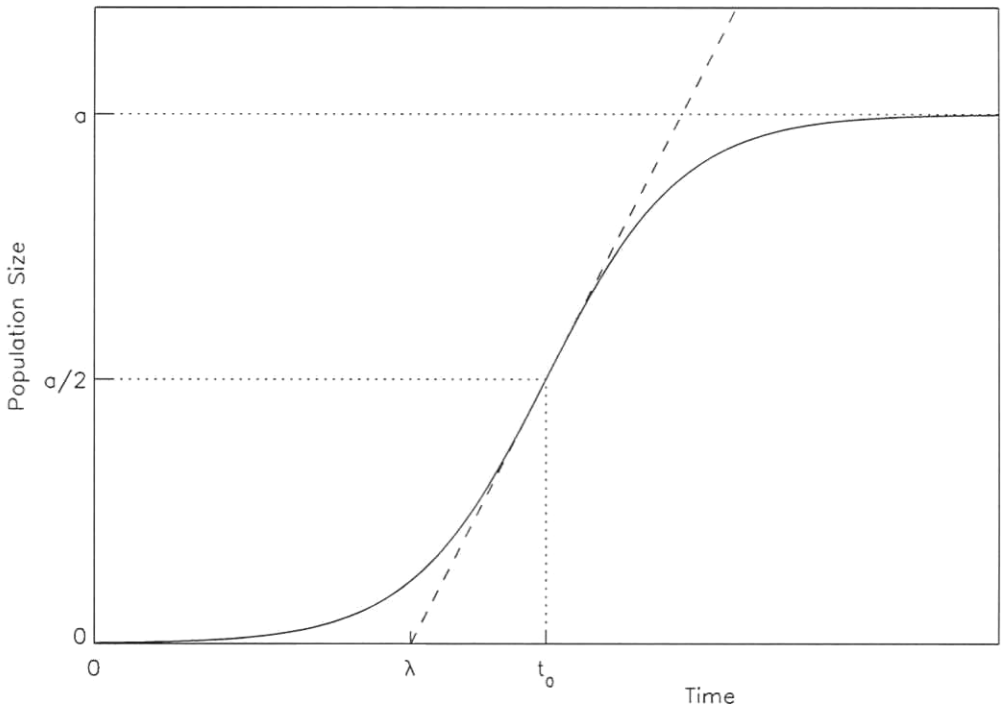


Figure 2.1 Sigmoid curve for calculating latent period (λ), and burst size (a) for single step growth data. Dashed line represents the maximum specific growth rate which occurs at time $t=t_0$ (Carey-Smith, 2004).

All of the phage counts were adjusted relative to the infected culture (Carlson and Miller, 1994) by subtracting the unabsorbed phages count (C) from the titre at each time step. The phage counts were then divided by the number of infected centres (IC), which are calculated by subtracting the unabsorbed phages from the initial phage concentration (P), to give the relative titre of phages at each time step. These calculations provide counts of the free phages from the untreated samples, and the total phages (free and intracellular). The proportion of absorbed phages (A) was calculated by dividing the difference in unabsorbed phages and the initial phage concentration (infected centres) by the initial phage concentration. The multiplicity of infection was estimated in two ways; dividing the initial phage concentration by the initial bacteria concentration (Cells), (MOI 1), or by subtracting the unabsorbed phage from the initial phage concentration then divide the number by the initial bacteria

concentration (MOI 2). To ensure that the number of infected cells represented the number of killed cells (IC 2), Poisson distribution logic can be used to formulate the equation IC 2 = (Cells) ($1-e^{-MOI\ 2}$). The sigmoid curve has been shown to closely represent the process of phage replication. The latent period and the burst size, the number of phages produced from each infected centre, can all be calculated from the sigmoid curve parameters (Goodridge et al., 2003) (Figure 2.1).

The sigmoid equation describes the population size as a function of time and is given by

$$y = \frac{a}{1+\exp\left[\frac{-(t-t_0)}{b}\right]}$$

Where ‘a’ is the asymptote (maximum value reached), ‘t_0’ is the position (time) of maximum slope and ‘b’ is the maximal value of increases (Zwietering et al., 1990). The maximum slope occurs when $t = t_0$ and this can be found by differentiating ‘y’ with respect to ‘t’ to obtain.

$$\frac{dy}{dt} = \frac{a\exp\left[\frac{-(t-t_0)}{b}\right]}{b\left[1+\exp\left(\frac{-(t-t_0)}{b}\right)\right]^2}$$

If $t = t_0$ then the slope will be

$$\frac{dy}{dt} = \frac{a}{4b}$$

From Figure 2.1, the lag time (latent period), λ, can be calculated as

$\lambda = t_0 - 2b$

2. 12 Restriction Digest Profile Analysis of *Bacillus* Phage

2.12.1 Rapid DNA Extraction (S. Ismail and C. Billington personal communication, 2007)

This method for rapid DNA extraction relies on having a high titre of phage for simultaneous PEG/NaCl precipitation. High titre stock of phages was prepared as described in section 2.7 with the pellet being resuspended in 0.5 ml of SM buffer and transferred into 1.5 ml eppendorf. To remove any bacterial DNA, 10 µg/ml DNase I and RNAse (Roche) was added to an eppendorf before incubating for 1 h at 37 °C.

To concentrate phage DNA, 0.5 M EDTA, 20% (w/v) of SDS, (Appendix A.6.3 and A.6.4) and 10 mg/ml Proteinase K (Roche) was added into an eppendorf to give a final concentration of 2.5 mM EDTA, 4% (w/v) SDS, 0.5 mg/ml Proteinase K. Overnight incubation at 56°C was required before adding 1/3 volume of 5 mM NaCl for helping precipitation. Extraction with equal amount of phenol-chloroform-isoamyl alcohol (25:24:1, Sigma). The samples were mixed for 5-10 min on a rotor at 40 rpm before centrifuging at 4800 x g for 10 min and the top phase collected between each phenol extraction. After extraction, equal amount of chloroform ($CHCl_3$) was added to the top phase and centrifuged at 4800 x g for 10 min before the top phase was removed to an eppendorf again. The DNA was precipitated by adding 1/10 volume of aqueous 3 M sodium acetate (Appendix A.6.5) and 2 volumes of 100% ethanol. The samples were mixed and left overnight at 4°C before centrifuging at 4800 x g for 10 min and washing the pellet in 500 µl of 70 % ethanol (Appeddix A.6.6). The eppendorfs were

inverted before another centrifugation at 4800 x g for 10 min. The pellet was air dried at room temperature for approximately 2 h (until the whiteness of pellet and ethanol drops disappeared) and resuspended in 50 to 100 µl of 50 mM Tris (Appendix A.6.7). The quality of DNA extracted was estimated by using a spectrophotometer (ND-1000, NanoDrop) by the ratios at 260/280 nm against the blank (i.e. 50 mM Tris). The quality of extracted DNA was also tested visually with electrophoresis on a 1% agarose gel (Appendix A.6.2) at 100 volts for approximately 60 min.

The extracted DNA was restricted with *Hin6I, TaqI, DralI, SSPI, Bsp143I* endonucleases to calculate genome size. The DNA was size-fractionated by electrophoresis at 70 volts for approximately 80 min, on a gel containing 1% agarose in 0.5 x TBE buffer (Appendix A.6.1). Rapid DNA extraction was also carried out with A5ll phage with same quality tests as it acts as a control in this experiment.

2.13 Stability During Frozen and Refrigerated Storage

LB broth, LB broth with 15 % (w/v) glycerol (Appendix A.1.4 and A.1.5), SM buffer and SM buffer with 1 % (w/v) gelatin were prepared as described in the Appendix A.1.1. All high-titre phage stocks were prepared and the titre of the phage stock was then enumerated by the overlay method with indicator host strain as described in section 2.9. A 1:10 dilution of phage stocks was made with the above broths in tin-foil wrapped sterile 25 ml universal bottles and kept at -80°C, 4°C and 22°C. Samples were collected at day 0, 7, and 30 and the titres of phage stock were then enumerated by the overlay method.

2.14 Effect of Chloroform on Phages

High titre phage stocks were prepared and enumerated as described in section 2.9. 1 ml of phage stock was transferred into sterile 3 ml bijou bottles and 1 drop of chloroform was added to each bottle. The solution was mixed gently and left on a rotor (40 rpm) for 30 min at room temperature before titring phage again to determine if chloroform affected viability.

2.15 Effect of Phage Treatment on Mashed Potatoes at 25, 10 and 4°C

Bacteria and phage were prepared as described in section 2.4.1 and 2.9, respectively. The bacteria and phage were diluted with BHI broth and SM buffer, respectively, as required to achieve a pre-determined ratio of phage to bacteria. 20 g of a mash potato mixture (Appendix) with 1.8 mM $MgSO_4$ and 0.8 mM $CaCl_2$ (Cooney, et al., 1975) was transferred into sterile 25 ml universal bottles with final concentrations of bacteria and phage of 10^8 cfu/ml or 10^6 cfu/ml and 10^8 pfu/ml to give phage to bacteria ratios of 1 and 1000 respectively Universal bottles were inverted and incubated in 25°C, 10°C and 4°C. The remaining volume of the phage dilution was stored on ice until assayed. The remaining bacteria suspensions were kept on ice for quantifying the number of cells present and to be used as the indicator culture for the plaque assay.

For the mixtures incubated at 25°C, samples were taken at time 0, 1, 2, 4 and 24 h, while other universal bottles that were incubated at 10°C and 5°C with sampling time of 0, 1, 2, 3 and 7 days. The quantification of cells in the samples was achieved by serial dilution by a factor of ten with BHI broth. All plates were incubated overnight at 37°C, before colonies were counted.

Chapter 3

Results

3.1 Isolation of *Bacillus cereus* From Environment

B. cereus was isolated from Cinderella instant mashed potatoes, and the isolates were purified by plating on Mannitol yolk polymyxin selective agar (Mossel et al., 1967). The isolates were then characterised and identified by rapid confirmatory test (staining test) (Holbrook and Anderson, 1980), and MYP test (Mossel et al., 1967; Donovan, 1958; Coliner, 1948), haemolysis test (sheep blood agar, Oxoid NZ Ltd), and using microscopy to confirm crystal formation (J. A. Hudson, personal communication, 2007) and ensure that isolates were not closely related species (i.e. *Bacillus thuringiensis*, *Bacillus subtilis* or *Bacillus anthracis*) (Table 3.1).

3.2 Isolation of Phages Infecting *Bacillus* Species

B. thuringiensis subsp. *israelensis* (NZRM 2981, BT), *B. cereus* (NZRM5, BC) and two isolates of *B. cereus* from mashed potatoes (Section 3.1: A2 & D2) were used as indicator hosts for phages in all samples tested. Forty soil, five milk, two rice and a spice sample were tested for the presence of phages. Isolation methods including combinations of temperature (incubation at 10, 24, and 37°C), agar concentrations (1, 0.8, 0.6, and 0.4 % agar in the overlays) or enrichment using all four indicator hosts and enrichment using the same broth with addition of 3% beef extract was used for each sample.

In total, four distinct plaque types were identified from two enriched soil samples. Two distinguishable plaques were found to form on *B. thuringiensis*. One was a tiny and clear

plaque that gave rise to a phage called FWLBt1, and the other was a small plaque with a turbid ring that gave rise to a phage called FWLBt2. Unfortunately, FWLBt phages were lost after storage at 4°C for a month in SM buffer. Therefore, no further work was carried out on these phages.

Table 3.1 Distinguishing characteristics of *B. cereus* isolates from Cinderella instant mashed potatoes in comparison with other *Bacillus* species from literatures (Todar, 2005; Cheun et al., 2001; Rhodemhamel et al., 1998).

	***B. cereus* (A2 &D2)**	***B. cereus***	***B. thuringiensis***	***B. subtilis***	***B. mycoides***	***B. anthracis***
Cell morphology	4-6 mm Cream/white	2-5 mm Cream/ white	2-5mm White & rough edge	3-5mm Cream/white	3-5mm rhizoid/hairy colony	3-5mm Grey/white, tubid ring
Spore stained	Green	Pale green/mid green	Green	Green	Green	Clear (resistant)
Lipid globule stained	Blue-black	Black	Black	Black	Black	Black
Vegetative cytoplasm stained	Red	Red	Red	Red	Red	Red
Mannitol test	-	-	-	-	+	-
Lecithinase test	+	+	+	-	+	+
Haemolysis (sheep blood plate)	+	+	+	+	+	-
Mobility	+	+	+	+	-	-
Crystal formation	-	-	+	-	-	-

Two plaque types were observed on hosts D2 and A2. D2 and A2 are assumed to derive from the same strain of *B. cereus* because they are indistinguishable by all tests described here. A tiny (1mm) and translucent plaque give rise to a purified phage called FWLBc1, and another tiny (1mm) and turbid plaque give rise to a purified phage called FWLBc2. The phage, from plaques, observed from the soil enrichment samples were propagated for purification and characterisation. The plaque types and morphology became more uniform with each passage, ultimately resulting in one plaque morphology on each plate. Neither phage produced turbid rings on Brain Heart Infusion (BHI) plates (Figure 3.1).

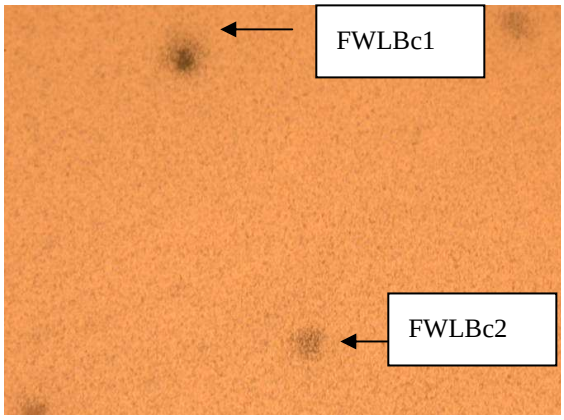


Figure 3.1 *B. cereus* phages from soil sample S23 on overlay plate. Soil samples were collected from various sites, i.e. gardens, composes, and parks within Christchurch by ESR staffs.

Two high titre stocks of FWLBc1 and FWLBc2 were prepared. These stocks had concentrations ranging from 10^8 to 10^{10} pfu/ml. These stocks were used for phage characterisations, restriction digest profiles, stability tests and other tests to distinguish if the phages differed from each other.

3.3 *Bacillus* Phage Characterisation

3.3.1 Transmission Electron Microscopy (TEM)

Both FWLBc1 and FWLBc2 phages were judged to belong to the *Myoviridae* family because of the appearance of contractile tails in the electron micrographs (Figure 3.2). FWLBc1 had an average head diameter of 97 nm +/- 5.0 (n=42), average tail length of 210 nm +/- 25 (n=42), average contracted sheath diameter of 23nm +/- 4.0 (n=42), and average tail tube diameter of 10 nm +/- 2.5 (n=42). Phage FWLBc2 had similar dimensions with an average head diameter of 96 nm +/- 6.0 (n=65), tail length of 219 nm +/- 10 (n=65), a contracted sheath diameter of 27 nm +/- 3.5 (n=65), and tail tube diameter of 10nm +/- 1.5 (n=65) as shown in Table 3.2.

Both FWLBc1 and FWLBc2 had an isometric head, rigid contractile tail, and a base plate with branch-like tail fibrils (Figure 3.2). In Figure 3.2, it is noticeable that some phage heads were white while others were dark. Dark heads are normally taken as evidence that some phages have either not packaged, or have lost, their nucleic acid genomes. Nevertheless, all the physical characteristics of FWLBc1 and FWLBc2 phages obtained from TEM are of the *Myoviridae* family (Table 3.2).

3.3.2 Host Range

The host ranges of *B. cereus* phages FWLBc1 and FWLBc2 were assessed by assaying against several *Bacillus* species and strains (Table 3.3). High titre stocks of phage were freshly prepared with the concentration of stocks measured and diluted to corresponding final titres of 10^3, 10^5, and 10^7 pfu/ml. Different plating efficiencies suggested that the phages were only marginally effective on some hosts and that both phages have narrow host ranges. Furthermore, FWLBc1 was lytic on 6 of the 12 hosts, while FWLBc2 was lytic on 9 of the 12 hosts, indicating a broader lytic range. However, the lysis activity on *B. cereus* (NCTC 11143,

NCTC 11145, and NZRM 2981) by FWLBc 2 phage was suspected to be lysis from without, meaning that lysis caused by adsorption of phage above a threshold value of phage concentration. The cell contents were liberated by a "non productive infection" of distension and destruction of the cell wall. Inhibition zones formed at the highest titre of phages and not at 1/10 or 1/100 dilutions of phage stocks may indicate of lysis from without (Figure 3.3).

FWLBc1 and FWLBc2 had different plaque-forming efficiencies. For example, for spot test on *B. mycoides* (from raw mussel) lawn, FWLBc1 phage formed plaques at lower titres (10^3 pfu/ml) while FWLBc2 could only form plaques at higher titers (10^5 pfu/ml). This type of lysis was caused by infection of the bacteria by a single phage particle and multiplication of this phage particle up to a threshold value. The cell contents are then liberated into solution without deformation of the cell wall. The *Bacillus* host isolated from Cinderella mash potatoes (D2) was chosen to be used in further characterization as it required a lower phage concentration for lysis and, therefore, it was easier to work with.

3.3.3 Growth curve of *Bacillus*

The relationship between the OD reading and *B. cereus* colony count was determined in order to find the time required to reach stationary phase. A growth curve of *B. cereus* (D2) was realized because a lower phage concentration was required for lysis from the host range experiments which is easier to work with.

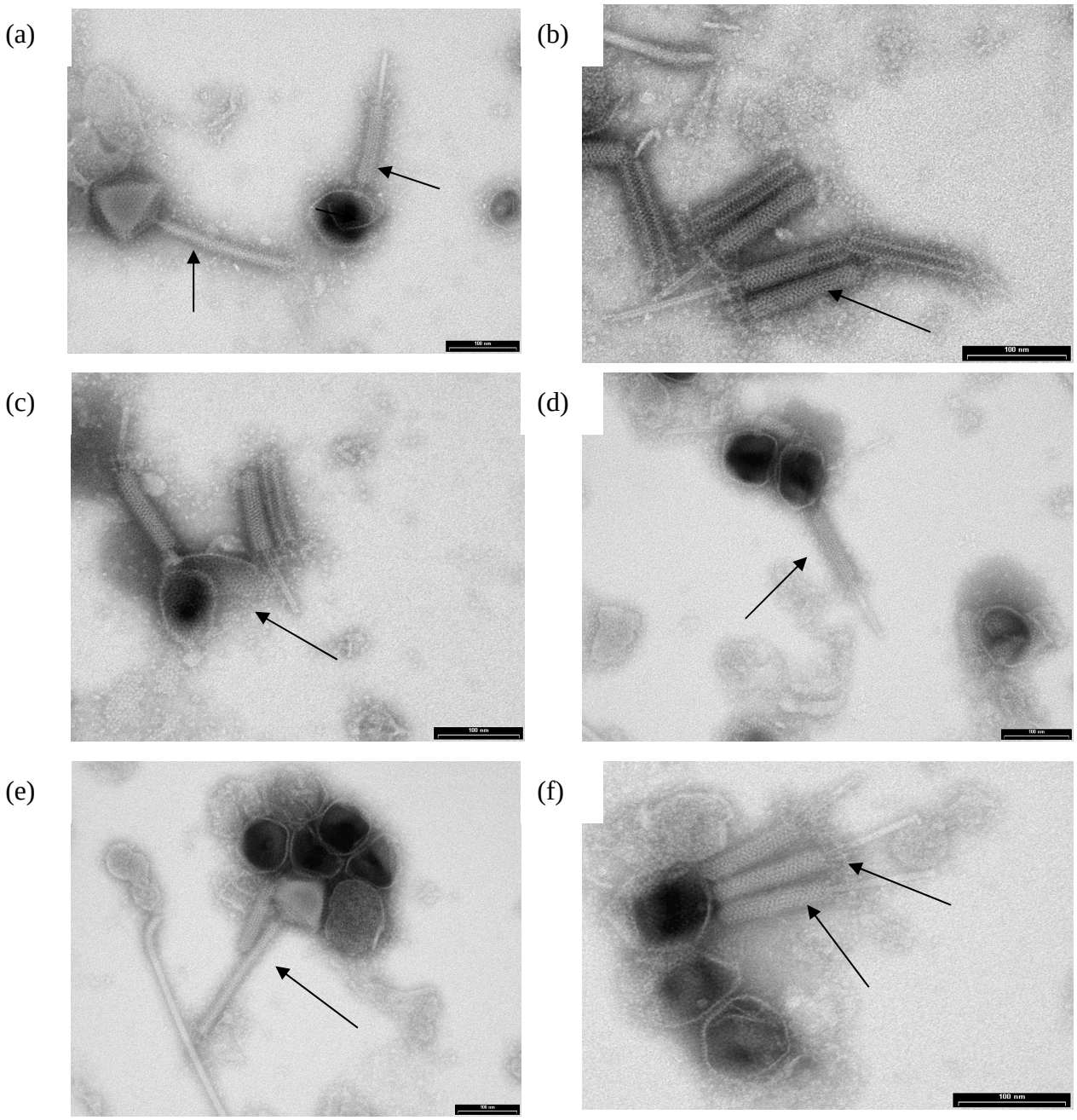


Figure 3.2 TEM of *Bacillus* phage (a) contracted and non-contracted tails of FWLBc1, (b) sheath and tail fibril of FWLBc1, (c) FWLBc1 with an open head (arrows), (d) FWLBc2 with contracted tail, (e) FWLBc2 with non-contracted tail and, (f) FWLBc2, contractile tail with tail fibril and tail tube shown (scale represents 100 nm, confirmed using catalase axles).

Table 3.2 Physical characteristics of *B. cereus* phages from TEM in comparison with *Myoviridae* family and other *Bacillus* phages in literature.

Physical characteristics	**FWLBc1**	**FWLBc2**	**SP50 and Bastille**	***Myoviridae* family**
Head diameter, nm (average)	97 ± 5.0	96 ± 6.0	About 90	53-160
Tail fibrils?	Yes	Yes	Yes	frequently with multiple tail fibers
Tail length, nm (average)	210 ± 25	219 ± 10	203	-
Sheath diameter, contracted, nm (average)	23 ± 4.0	27± 3.5	22	about 24
Tail tube, diameter, nm (average)	10 ± 2.5	10 ± 1.5	9	7 to 10
Contractile tail?	Yes	Yes	yes	Yes
Number of phages measured	42	65		-

Initially, BHI broth was inoculated with a fresh colony of *B. cereus* and incubated at its optimal growth temperature. Although the OD reading of the culture was described over time as a sigmoid curve, no reliable relationship between OD reading and colony count was found (data not shown). This non-reliable relationship might be a result of the interference of the spores of *B. cereus*. In order to get a reliable relationship between OD readings and colony forming units, fresh BHI was inoculated with 100 µl saturated culture of *B. cereus* and incubated at the optimal growth temperature. When *B. cereus* had reached its optimal growth rate (approximately 3 h after inoculation), subsequent of 2-fold dilutions (i.e. 50:50) of this culture were made with fresh BHI broth. OD reading and colony count were determined for each dilution to find the linear relationship of OD reading and colony count. The linear

relationship was obtained as y = 4.4169 x + 0.0959, with vertical axis representing host concentrations (10^8 cfu/ml) and horizontal axis being the OD measurements. It was noted that the linear relation had R^2=0.9493, indicated 95% of confidence (Figure 3.4). From this relationship, it was suggested that approximate 3.6 x 10^8 cfu/ml of *B. cereus* bacteria are presented in the broth when the OD reading was 0.8.

Table 3.3 Host range of *Bacillus* phages FWLBc1 and FWLBc2

Indicator hosts	**FWLBc1 (pfu/ml)**	**FWLBc2 (pfu/ml)**
A2 (*B. cereus* from potato)	(10^5)+	(10^3)+
D2 (*B. cereus* from potato)	(10^3)+	(10^3)+
B. cereus (NCTC 11143)	-	(10^7)**
B. cereus (NCTC 11145)	-	(10^7)**
B. cereus (NZRM5)	(10^7)+	(10^7)**
B. thuringiensis (NZRM2981)	-	-
B. thuringiensis (DSM 2046)	(10^3)+	(10^3)+
B. licheniformis (DSM 603)	-	(10^7)*
B. subtilis (NTCT 3610)	-	-
B. mycoides (ATCC 6462)	(10^7)*	(10^7)*
B. mycoides (from raw mussel)	(10^3)+	(10^5)+
B. megaterium (NCTC 10343)	-	-

Starting titre for FWLBc1: 1.36 x 10^8 pfu/ml, FWLBc2: 7.4 x 10^{10} pfu/ml,

+ distinct clear plaques, * host obviously reduced but no distinct plaques, ** likely to be lysis from without, lysis activity observed with highest phage concentration shown in brackets. Pfu numbers are determined by plating on each specified host that indicates an efficiency of plating measure.

3.3.4 Lysis Ability of *Bacillus* phages

OD measurements were used as a quantity indicator of *B. cereus* present in the broth, based on the linear relationship found previously. The average number of bacteria was described over time as a sigmoid curve (n=8), showing a lag, exponential, and stationary phases over time. However, addition of either FWLBc1 or FWLBc2 phage caused lysis of the host to occur (Figure 3.5). Each phage's ability to lyse the host was also assessed with two different phage to host ratios and the host control.

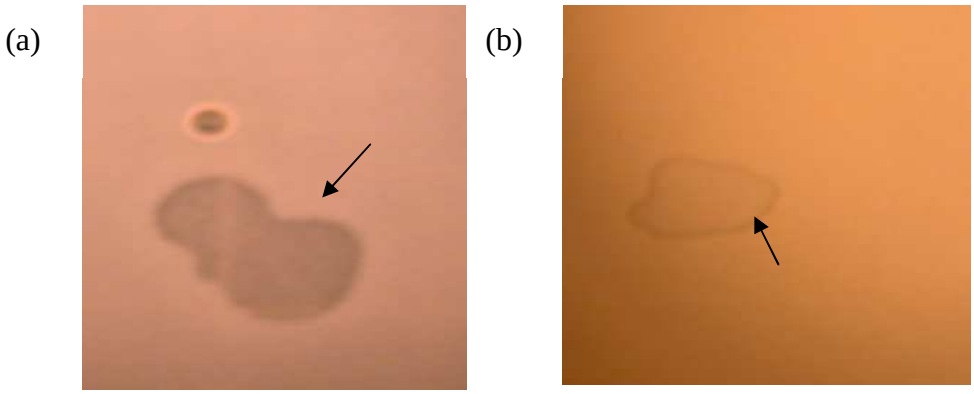


Figure 3.3 Lysis of *B. cereus on* BHI plates (a) FWLBc2 phage (10^3 pfu/ml) on *B. cereus* (isolated from Cinderella mashed potato) host lawn and (b) FWLBc2 phage (10^7 pfu/ml) on *B. cereus* (NZRM5) host lawn, which is likely to be lysis from without.

The multiplicity of infection (MOI) was calculated as the initial phage concentration divided by the initial concentration of bacteria. In Figure 3.5 (a), MOIs of FWLBc1 were calculated to be 0.38 and 0.03. With 10 times difference in the MOI, the higher MOI appeared to lysis *B. cereus* in approximately 120 min after infection, while the lower MOI took 150 min to reduce the number of colony forming units. For FWLBc2 (Figure 3.5 b), the MOI values were calculated to be 0.58 and 0.01. With approximately 50 times difference in phage concentration, the higher phage to host ratio took approximately 120 min while lower ratio took longer time

(approximately 150 min) to initiate the lysis of *B. cereus* host.

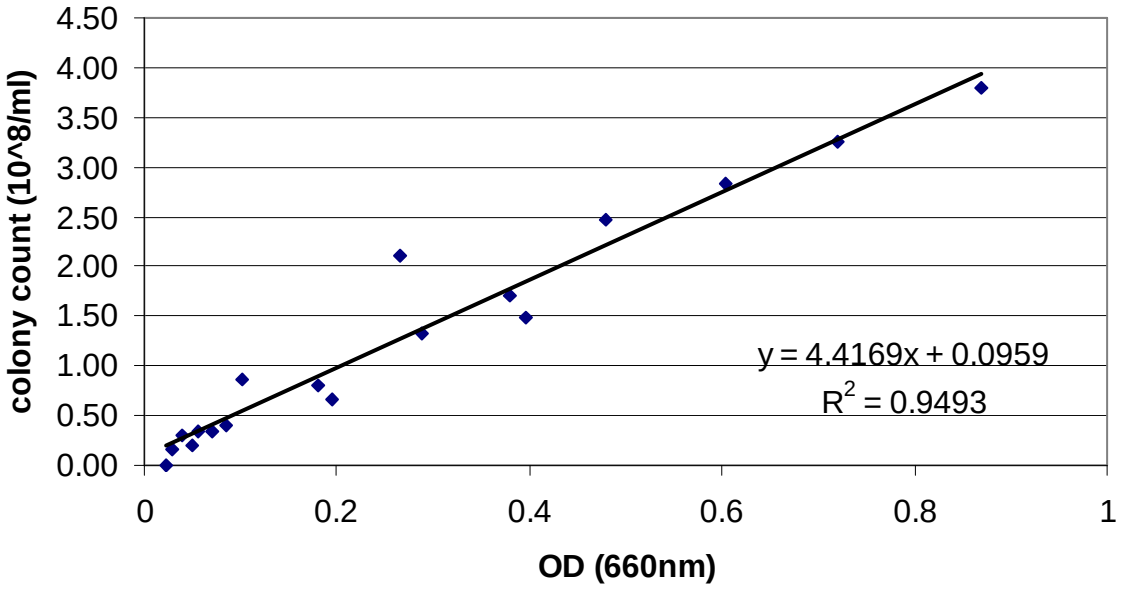
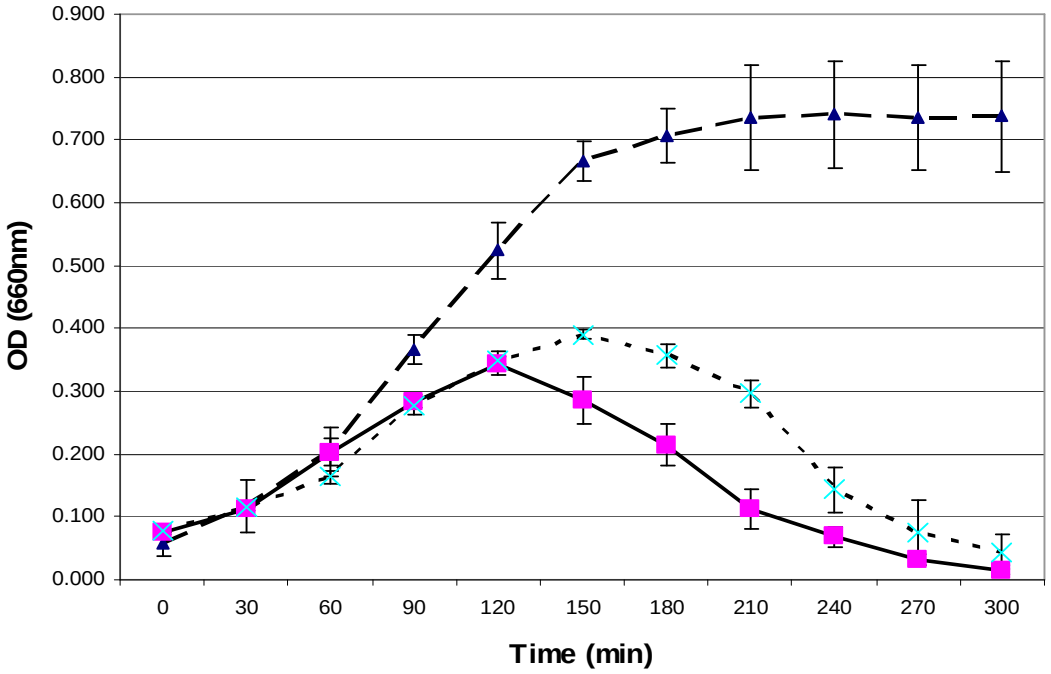


Figure 3.4 Relationship of OD at 660 nm and colony count of *B. cereus* using saturated culture and 2-fold dilution technique.

Both FWLBc phages initiated the lysis of host at, approximately, the same time regardless of the MOI values, which may indicates these two phages have similar lysis activity on *B. cereus* host. However, shorter time (195 min and 255 min for lower and higher MOI respectively) was required for FWLBc2 to reduce the OD reading back to its original value (OD= 0.07). In consideration of different ratios of FWLBc phages to host, this may indicate that increasing lysis activity of *B. cereus* host were found with increasing ratio of phage to host concentration (MOI value).

(a)



(b)

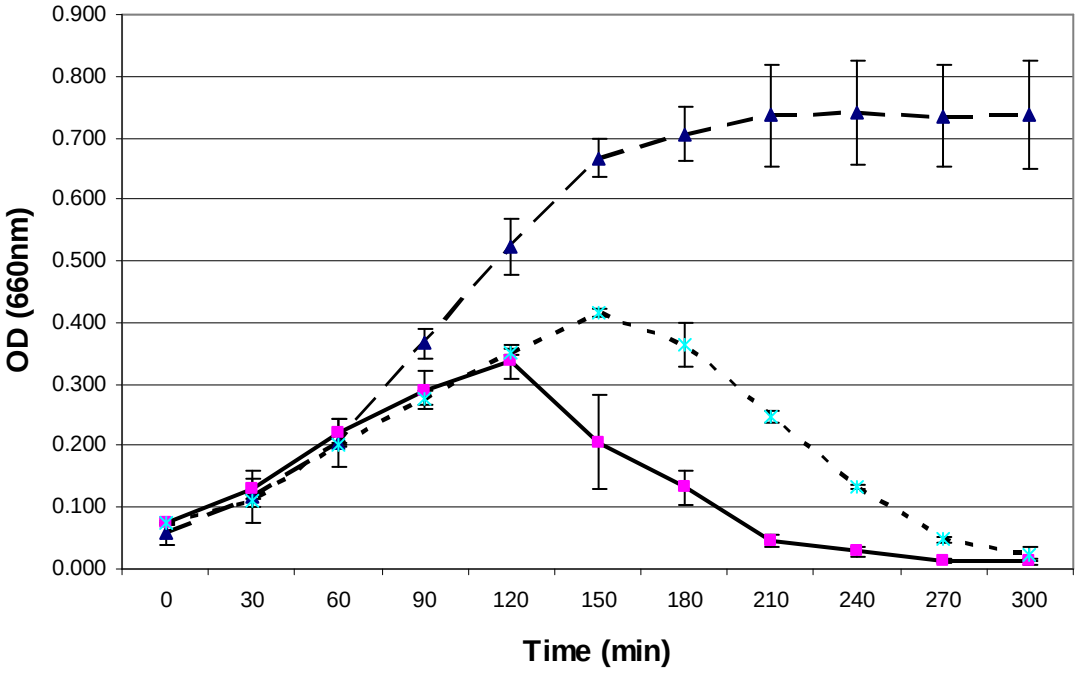


Figure 3.5 Lysis ability of *Bacillus* phage on *B. cereus* host isolated from Cinderella mashed potato (a) FWLBc1 and (b) FWLBc2. Dash line represent control culture of *B. cereus*, solid line represent *B. cereus* with higher MOI (0.38 and 0.58 for FWLBc1 and FWLBc2 respectively), and the dotted line represents *B. cereus* host with lower MOI (0.03 and 0.01 for FWLBc1 and FWLBc2, respectively).

3.3.5 Single Step Growth Curve

The single step growth experiments were conducted using D2 and FWLBc phages at 37°C in BHI broth in aerobic conditions. The burst size and latent period for FWLBc1 and FWLBc2 were calculated from the sigmoid curve drawn by SigmaPlot (SigmaPlot 9.0, Systat Software Inc.) (Figure 3.6). The latent period (λ) is the period between phage adsorption and cell lysis. It was determined by subtracting twice the maximum specific growth rate (b) from the time of maximum slope plus 15 minutes, to account for the centrifugation time. The burst size (a) is the number of phages produced by an infected cell, which was directly obtained from a SigmaPlot parameter.

FWLBc1 had a burst size of 322 pfu (+/- 20 pfu) and a latent period of 93 min. (+/- 3min). The fraction of phage adsorbed was obtained by subtracting the concentration of unadsorbed phage from the initial phage concentration, and then dividing that number by the initial phage concentration, and was calculated to be 0.99 within 5 min. This indicates phage adsorption was efficient under the experimental conditions and was confirmed by calculating MOI to be 0.11 since both the ratio of initial phage concentration and initial bacterial concentration (MOI 1), and the ratio of difference of initial phage and unabsorbed phage and the initial bacteria concentration (MOI 2) are similar under the experimental conditions. In addition, values for determining the productively infected cells (IC 1) and the estimation of cells killed by the phages (IC 2) were found to be similar (i.e. 1.0 $x10^6$ and 8 x 10^6 cfu respectively), this suggested that both phage and bacteria were viable at time of infection.

FWLBc2 had a burst size of 300 pfu (+/- 8 pfu) and a latent period of 84 min (+/- 2min). The fraction of phage adsorbed within 5 min was calculated to be 0.98, and was confirmed with same calculated MOI values of 0.33 (i.e. MOI 1 and MOI 2). There was approximately a

10-fold difference between productively infected cells (IC 1= 2 x 10^5 cfu) and the estimated value of cells killed by the phages (IC 2 = 5.7x 10^6 cfu) indicating that conditions for infection were not optimum. However, the fraction of phage adsorbed indicates that phage adsorption was efficient in this experiment.

3.3.6 Restriction Enzyme Digestion Profiles

Restriction enzyme digestion profiles of FWLBc phages were conducted to determine their genome size which could assist with further characterisation. Both a NanoDrop Spectrophotometer (data not shown) and agarose gel electrophoresis (Figure 3.7 a) were used to determine the quality of the DNA extracted. The ratios at 260/280 nm were close to 1.8 as determined by the NanoDrop spectrophotometer, indicating that extracted DNA was likely to be free of contaminating protein. The DNA extracted from both FWLBc1 and FWLBc2 phages migrated as one distinct band along with the same quantity of purchased control phage lambda (λ) DNA on a 1% agarose gel (Figure 3.7 a). This indicated that the extracted DNA was pure and contained no DNA or plasmid from the bacterial chromosome, as three defined bands (linear, open circular and supercoiled) will be presented on the agarose gel if plasmid was presented.

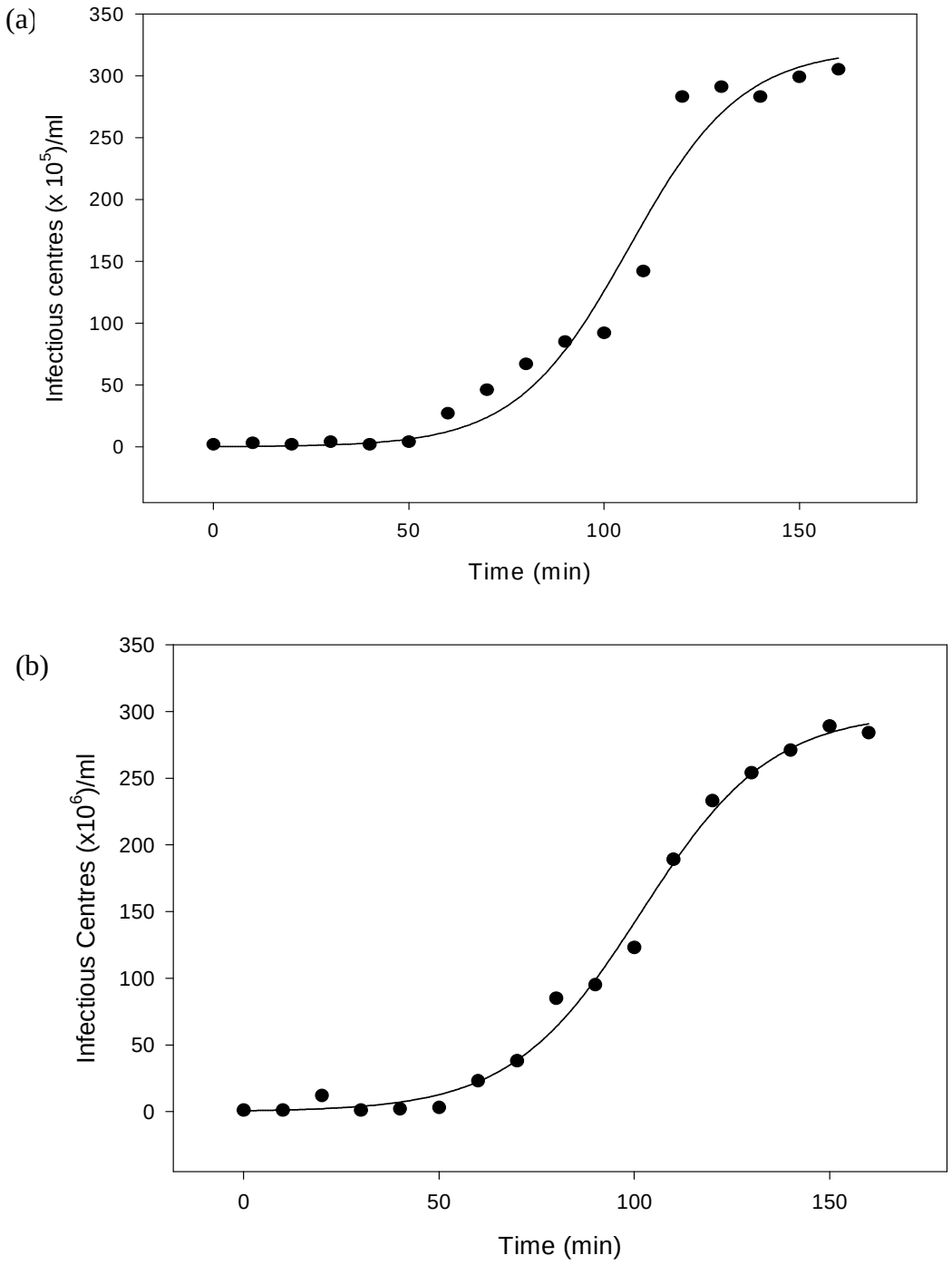


Figure 3.6 Single Step Growth of *Bacillus* phage (a) FWLBc1 and (b) FWLBc2 (host D2) at 37°C. Circles represent free phages enumerated and phage of the supernatant after a 15 min centrifugation and the sigmoid curve on phage production without unadsorbed phages is shown on the graph (R^2= 0.97 and 0.99 for FWLBc1 and FWLBc2, respectively).

The extracted DNA of both phages was then treated with restriction enzymes *Hin6I*, *SSpI*, *DraI*, *TaqI* and *Bsp143I*. However, neither of the extracted phage DNA was susceptible to *Bsp143I* (data not shown). *Hin6I*, *SSPI*, *DraI* and *TaqI* enzymes yielded very similar patterns for phage FWLBc1 and FWLBc2 on a 1% agarose gel (Figure 3.7 c). These results indicated that the two phages had similar sized and possibly closely related genomes. Both FWLBc1 and FWLBc2 phages have calculated genome sizes of approximately 134 kb, as determined by combining the fragment sizes obtained with the above four enzymes.

The extraction method used was an improved alternative developed by ESR, with slight deviation from standard extraction method. A511, a well-studied *L. monocytogenes* phage of the *Myoviridae* family, was used as a control to test the extraction method. A511 (Zink and Loessner, 1992) was extracted by the method (Section 2.13) used with the FWLBc phages. The ratio at 260/280 nm of extracted A511 DNA (i.e. 1.75) determined by the NanoDrop spectrophotometer indicates that it was a high quality DNA preparation. The DNA extracted from A511 phage migrated as one distinct band along with the control phage lambda (λ) DNA on a 1% agarose gel (Figure 3.7 b) further supporting the conclusion that the extraction method conduced in this study was pure. The phages of the *Myoviridae* family could be especially difficult to digest (O'Flynn et al., 2004), however, this was not the case in the study as A511 DNA was reported to be digested by many restriction enzymes (Loessner et al., 1994). However, here only *KpnI* and *NcoI* were used due to the lack of available restriction enzymes. The smear and a thick band (Figure 3.7 c) at just less than 12 kb in the gel indicated some chromosomal DNA might be present and the DNA was only partially digested, which might leads to smaller bands not easily visible from the digest.

As observed from Figure 3.7 (c), the extracted A511 DNA was not purely phage DNA, which limited the role of A511 to act as a control; therefore, cannot support that the DNA extraction method on this particular gel was efficient. It was suspected that contamination might have occurred when extracted A511 phage was digested with restriction enzyme. While time did not permit further repetition of the extraction and digestion process, the single band on 1% agarose gel from figure 3.7 (a) and (b), along with 260/280 ratio on Nanodrop spectropotometer indicated the efficiency of DNA extraction method used for the *B. cereus* phages. Moreover, the thick band which was presented in undigested FWLBc phages (Figure 3.7 c, lanes 2 and 8) was absent in lanes 3-6 and 9-12, instead, smaller DNA fragments of bands were present in digested FWLBc1 and FWLBc2, indicated that digestion of restriction enzyme with FWLBc phages were efficient.

3.3.7 Stability During Frozen and Refrigerated Storage

The stability of phage under refrigerated and frozen conditions could assist with further characterisation of the phage and help to determine the best storage condition for future study. FWLBc1 and FWLBc2 were stored in LB broth, SM broth, SM broth + gelatin (2%) and LB broth + glycerol (15%) at 22, 4 and -80°C. After 7 days of storage, 10 to 100 folds of phage concentration reduction was found with all FWLBc1 broths stored at -80°C, whereas no obvious reduction in the phage concentration was found in other storage temperatures. After 30 days of storage at -80°C, phage FWLBc1 stored in SM broth and SM broth + gelatin (2%) broth could not be revived, while LB broth and LB broth + glycerol (15%) retained 100 and 10-fold of the initial phage concentration. After 30 days of storage, there was no reduction in titre for phage stored in SM broth at 4°C, while other storage conditions resulted in a 10 to 1000 folds of reduction in phage concentration.

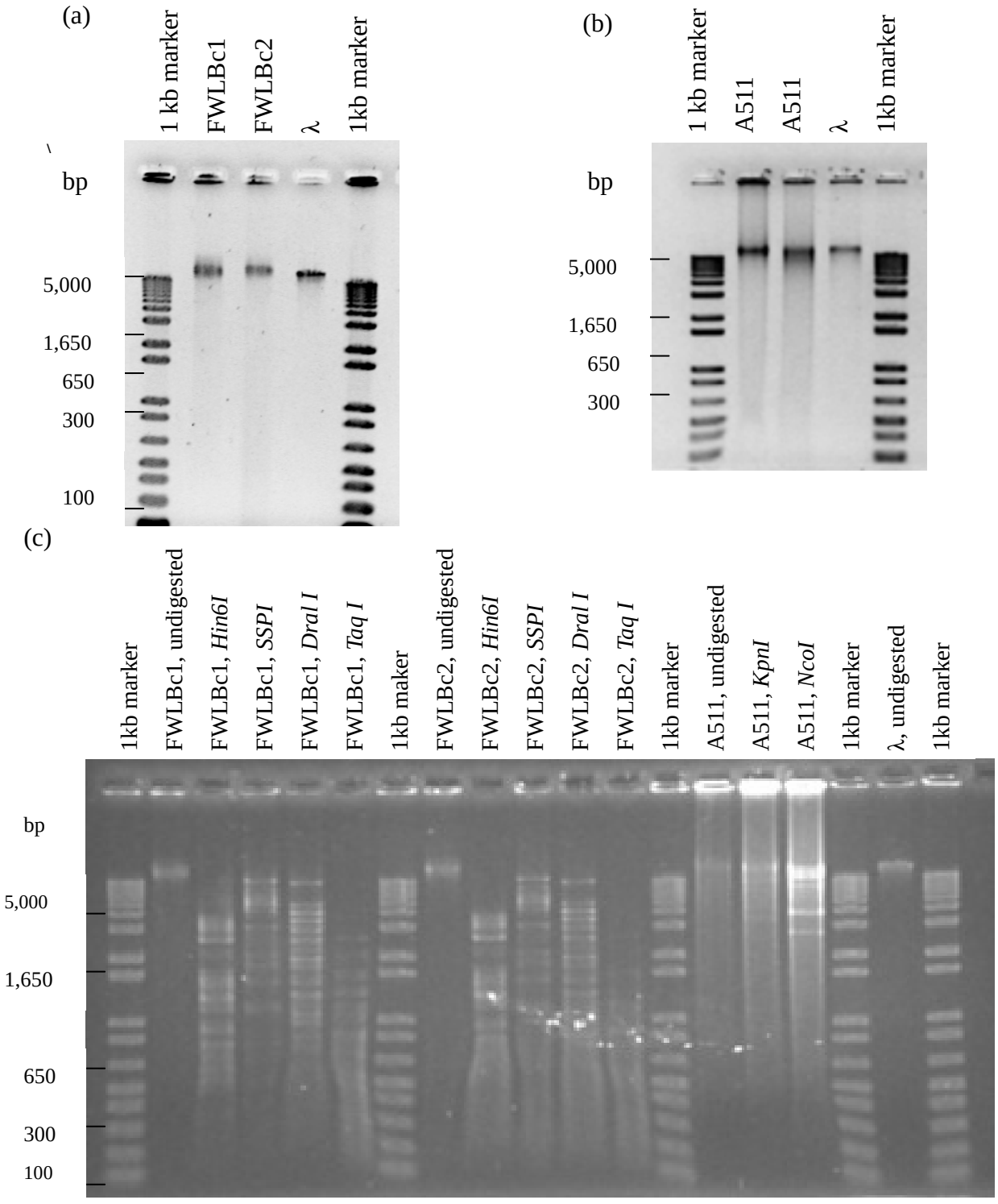


Figure 3.7 (a) Distinct single band of extracted FWLBc phages DNA in comparison to control phage lambda (λ) DNA on 1% agarose gel. (b) Distinct single band of extracted A511 phage DNA in comparison with control phage lambda (λ) DNA on 1% agarose gel. (c)

Restriction enzyme digestion patterns of FWLBc1 (lane 2-6), FWLBc2 (lane 8-12) and LM A511 (lane 14-16). FWLBc genomic DNAs were extracted and digested with restriction endonucleases *Hin6I*, *SSPI*, *DraI* and *TaqI*, and analyzes on a 1% agarose gel. Size markers included a 1 kb DNA marker (Invitrogen), with undigested λ genomic DNA as a control with undigested FWLBc phages.

FWLBc2 could be stored in LB broth or SM broth + gelatin (2%) at 4°C for at least 7 days without any reduction in titre, while other storage conditions showed a 10 fold of reduction. FWLBc2 stored in SM broth or SM broth + gelatin at -80°C could not be revived after 30 days. Phage concentration was reduced by 10 to 100 folds for FWLBc2 stored in LB broth + glycerol and LB broth at -80°C from its original titre. After 30 days of storage, best stability of FWLBc2 phage was observed in SM broth + gelatin at 4°C, when other storage conditions showed a 100 to 1000 folds of phage concentration reduction in titre reduction from the initial phage concentration.. High titre stocks of FWLBc1 and FWLBc2 with 10^8 to 10^9 pfu/ml were then stored at SM broth and SM broth + gelatin at 4°C respectively for later use.

3.3.8 Effect of Chloroform

The chloroform resistance of phage could add on to the knowledge of phage biochemistry and hence the characterization of phage. Fresh high titre stocks of FWLBc phages were prepared. A drop of chloroform was applied to each stock of phage. Both FWLBc1 and FWLBc2 phage were able to remind the same phage concentrations after exposed to chloroform for 30 min at 22°C. This indicated the presence of chloroform had no effect on both *B. cereus* phages isolated because most phages responded immediately (within few seconds) when chloroform was applied and therefore, chloroform resistant (Alatossava and Klaenhammer, 1991; Oh et al., 1999).

3.4 Effect of Phage Treatment on Mashed Potatoes at 25, 10 and 4°C

Figure 3.8 shows that the level of *B. cereus* in the inoculated Cinderella mashed potatoes increased significantly within 24 h with the application of neither phage stocks with incubation at 25°C. When phages were applied to *B. cereus* in sterile Cinderella mashed potato and incubated at 25°C at a phage to host ratio of 1:1, there was only a slight reduction in the pathogen numbers compared to the uninfected control. However, when this ratio was increased to 1000:1 (approximately 10^9 pfu/ml) the decline in pathogen numbers was rapid at approximately 2 h after application, and sustained throughout the course of experiment. Furthermore, the phage titre of FWLBc1 and FWLBc2 in Cinderella mashed potatoes were about 8.73 x 10^8 ± 0.16 pfu/ml and 4.35 x 10^8x 10^2 ± 0.68 pfu/ml respectively for a phage to host ratio of 1 and 1000 at 24 h. Phage was not present in the control culture of *B. cereus* at the end of incubation which indicated that the *B. cereus* phages remained active throughout the course of experiment and no contaminations of phage were present in the control group.

Phage treatment was then repeated at a phage to host ratio of 1:1 for both FWLBc1 and FWLBc2 at 10 and 4°C. No significant suppression of *B. cereus* numbers was observed with phage treatment in comparison to the control under these conditions (data not shown). It is also noted that this particular *B. cereus* host only grow to 10^1 cfu/ml in these conditions.

3.5 Isolation of Phage Infecting *Listeria* Species

L. monocytogenes (LM) strain 2000/47 (an ESR isolate provided by Pat Short at ESR, KSC) and strain 3009 (provided by Fonterra) were purified by plating on the PALCAM (Van Netten et al., 1989) selective agar. These two *L. monocytogenes* isolates were used as indicators hosts for the isolation of *Listeria* phages. Several animal sewage, effluent, ruminant faeces and compost samples (provided by ESR staffs, PPCS Belfast and Tegel Hornby) were tested for

the presence of phages (Table 3.4). The standard method of phage assay was used, a 0.7 % soft agar and incubation at 30°C overnight. However, the phage assay was later modified by using a much purer agarose, decreased concentration of 0.5%, and plates were incubated at 22°C overnight. More than one isolation method of direct isolation (Serwer et al., 2004) or enrichment was used for each sample.

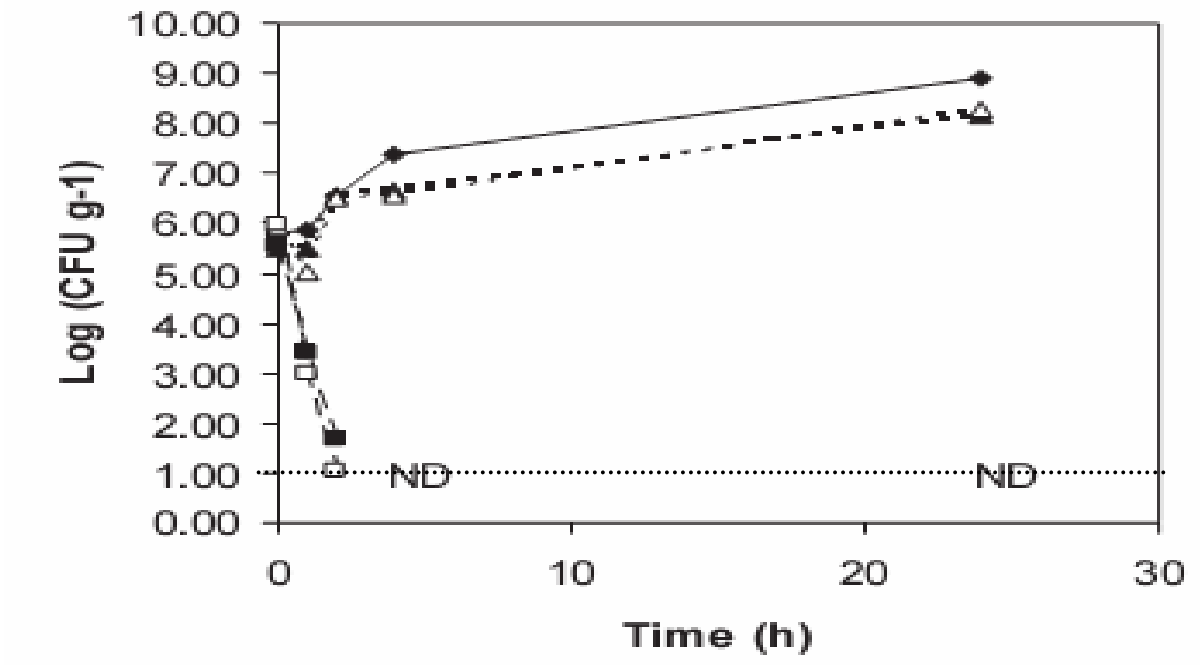


Figure 3.8 Reduction in counts of *B. cereus* incubated at 25°C in Cinderella mashed potato. Bc1 and Bc2 refer to the two phage FWLBc1 and FWLBc2. Note the rapid >4 log 10 reduction in numbers with phages added at approximate 10^9 pfu/ml. Solid line represented the number of *B. cereus* host without addition of phage. Dotted lines represented the number of *B. cereus* with phage to host ratio of 1:1 for FWLBc1 (shaded triangle) and FWLBc2 (non-shaded triangle). Dashed lines represented the number of *B. cereus* with phage to host ratio of 1000:1 for FWLBc1 (shaded square) and FWLBc2 (non-shaded square). The ND line represented the level of *B. cereus* numbers that were not detectable.

After using immunogenic separation (IMS) (C. Billington, personal communication, 2008), two plaques were observed from ruminant faecal samples. However, both of them were lost in the purification procedure. Therefore, further isolation methods were attempted. In total, five distinct plaques were isolated from two enriched ruminant faecal samples (i.e. RF5 and RF10), which gave rise to five phages, distinguished by the type of bacteria they attached to and the morphology of plaques. Four plaques were formed on LM 2000/47 host. A tiny and clear plaque (0.5 mm) gave rise to a phage called FWLLm1, while another, a tiny (0.5 mm) and translucent plaque gave rise to a phage called FWLLm2. Both phages were isolated from one of the ruminant faeces samples (i.e. RF5). Of the remaining two plaques formed, one was a small (1 mm) and turbid plaque which gave rise to a phage called FWLLm3 and the forth was a tiny (0.5 mm) and clear plaque which gave rise to a phage called FWLLm4. These two phages came from a different ruminant faeces sample (i.e. RF10) from the first two. One plaque was isolated on LM 3009 and appeared as a small (1 mm) and turbid plaque that gave rise to a phage called FWLLm5 and isolated from sample RF5. All five FWLLm phages were carried on for further characterisation.

3.6 *Listeria* Phage Characterisation

3.6.1 Transmission Electron Microscopy (TEM)

All five FWLLm phages were judged to belong to the *Myoviridae* family **(**Ackermann, 2006**)** because of the characteristic of their neck and contractile tail as shown in electron micrographs (Figure 3.9). FWLLm phages isolated could be classified into three types according to their physical characteristics from TEM and their host specificity (Table 3.5). FWLLm1, FWLLm2, and FWLLm4 were grouped as “type one” LM phage due to their similarity in size. FWLLm3 was classified as “type two” LM phage because its head size was bigger than the other FWLLm phages. FWLLm5 was classified as “type three” LM phage by

its host specificity (i.e. lyses to LM 3009 rather than LM 2000/47 like others).

Table 3.4 Number and type of samples tested for *Listeria* specific phages

	CONDITIONS							
Incubation Temperature	**30°C**						**22°C**	
Sample type	Direct isolation	Direct isolation (3% (w/v) beef extract)	Direct isolation (enrichment)	IMS	Enrichment (3% (w/v)beef extract)	Enrichment (Fraser selective supplement)	0.5% agarose	Total number of samples tested
Compost	4	4	4	4	4	4	8	8
Animal sewage	12	12	12	18	18	18	26	26
Ruminant faeces	2	2	2	6	6	6	12	12
Effluent	5	5	5	8	8	8	13	13
Total number of samples								59

Table 3.5 Physical characteristics of *Listeria* phages from TEM in comparison with *Myoviridae* family (Ackermann, 2006).

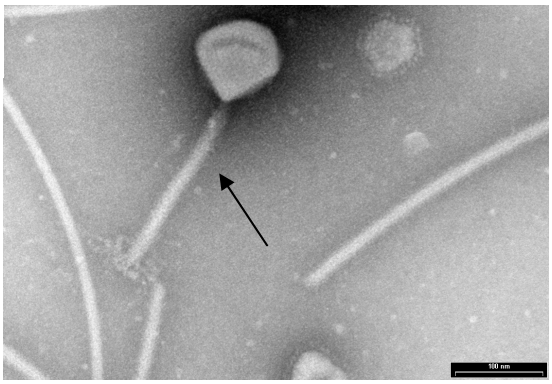
Physical characteristics	**Type one**			**Type Two**	**Type Three**		***Myoviridae* family**
	Lm1	**Lm2**	**Lm4**	**Lm3**	**Lm5**	**A511**	
Head diameter, nm (average)	98 ± 5.7 (22)	98 ± 9.3 (20)	96 ± 11 (31)	108 ± 11 (27)	100 ± 6.6 (32)	98 ± 5.3 (32)	53-160
Tail length, nm (average)	225 ± 19 (22)	222 ± 35 (20)	218 ± 22 (31)	226 ± 14 (27)	213 ± 61 (30)	210 ± 12 (4)	-
Sheath, contracted, diameter, nm (average)	18 ± 0.1 (3)	22 ± 0 (1)	20 ± 1.8 (8)	22 ± 4.0 (10)	17 ± 3.5 (27)	20 ± 2.2 (4)	about 24
Tail tube, diameter, nm (average)	8.4 ± 3.1 (19)	8.4 ± 0.9 (24)	8.9 ± 0.4 (26)	9.3 ± 2.7 (17)	8.0 ± 1.3 (6)	8.4 ± 0.9 (25)	7 to 10

Number in bracket is the number of phage measured for each characteristic. Lm1, Lm2, Lm3, Lm4, Lm5 refers to five phages of FWLLm1, FWLLm2, FWLLm3, FWLLm4 and FWLLm5.

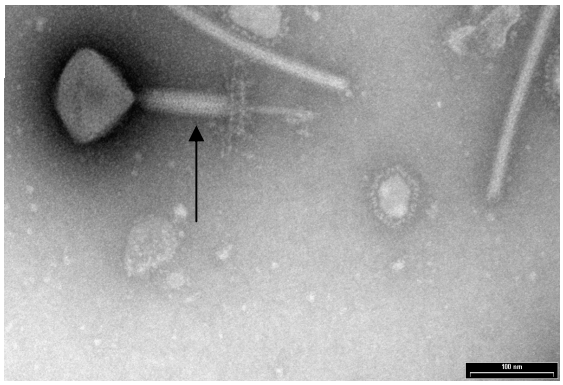
All five FWLLm phages had an isometric head, rigid contractile tail, and a base plate with branch-like tail fibrils as observed in the TEM. Some phage heads were white while others appear dark. This is normally taken as evidence that some phages have either not packaged, or have lost, nucleic acid genomes (dark head). Type one LM phages have an average head diameter of 98 nm ± 8.3 (n=73), tail lengths of 219 nm ±6.7 (n= 12), and tail diameters of 8.6

nm ± 1.5 (n= 69). Type two LM phage, FWLLm3, had on an average a larger head of 108 nm ± 10.6 (n=27) with tail length of 209 nm ± 4.9 (n= 10), and tail diameter of 9.3 nm ±2.7 (n=17). Type three LM phage, FWLLm5, had dimensions similar to type one LM phages, with an average head diameter of 100 nm ± 6.6 (n=32), tail length of 17.3 nm ± 3.5 (n=27), and tail diameter of 8.0 nm ± 1.3 (n=6)

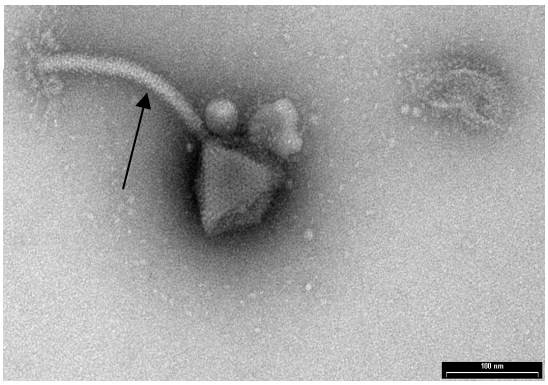
(a)



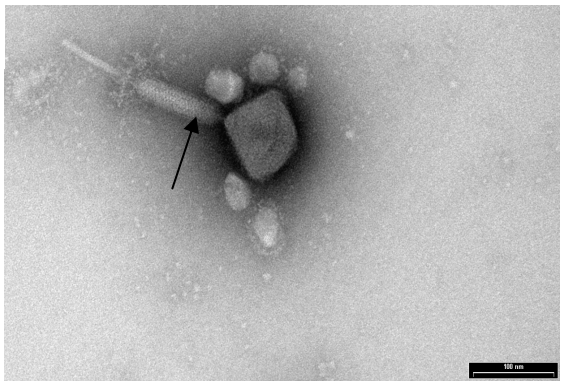
(b)



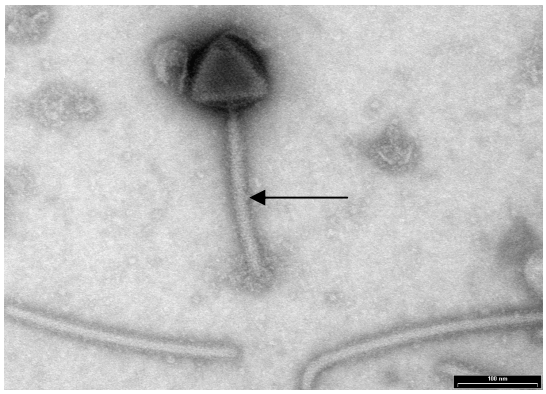
(c)



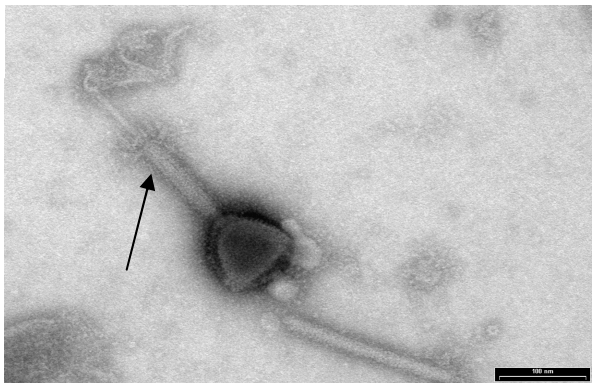
(d)



(e)



(f)



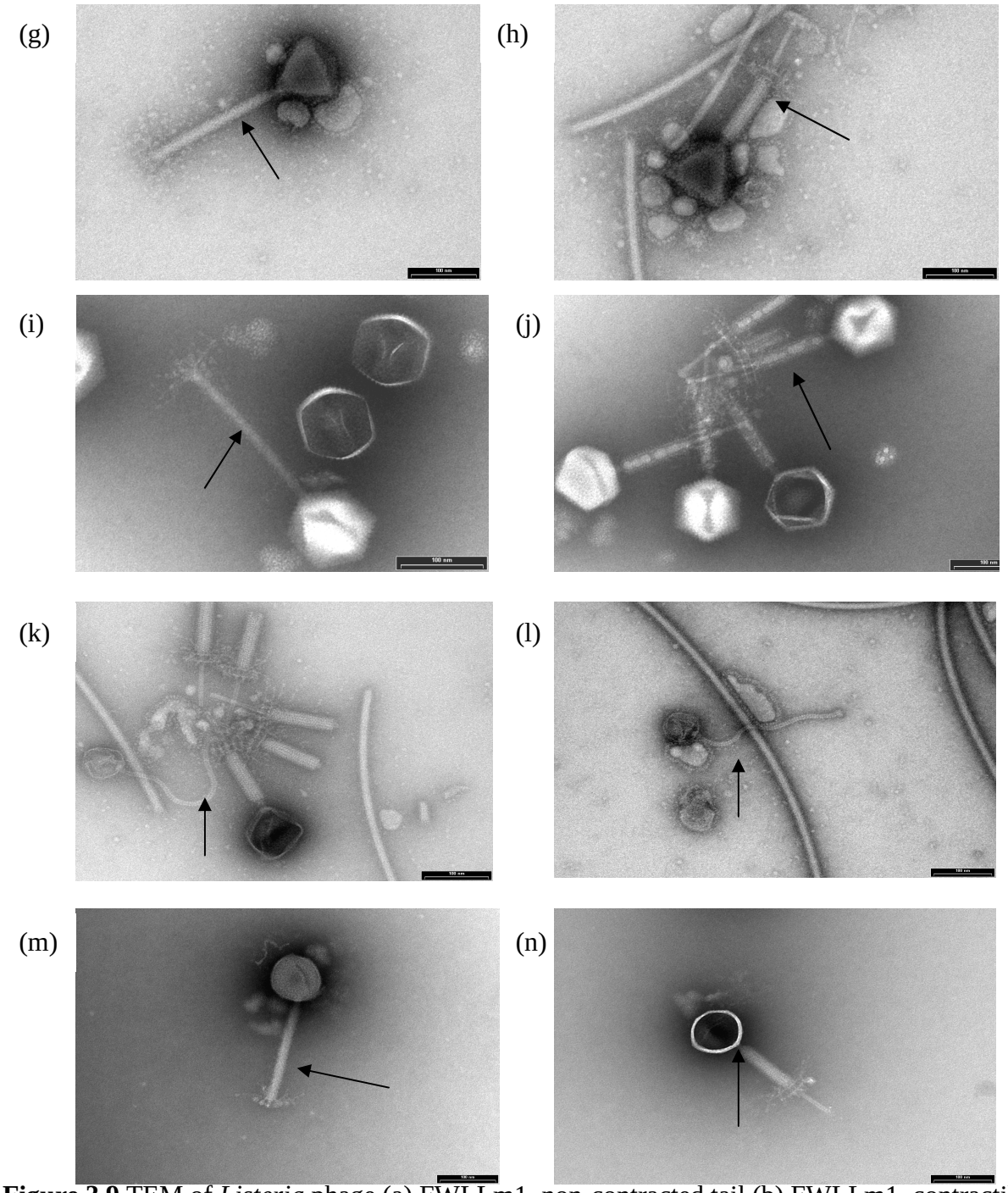


Figure 3.9 TEM of *Listeria* phage (a) FWLLm1, non-contracted tail (b) FWLLm1, contractile tail. (c) FWLLm2, non-contracted tail. (d) FWLLm2, contracted tail. (e) FWLLm3, non-contracted tail. (f) FWLLm3, contracted tail. (g) FWLLm4, non-contracted tail. (h) FWLLm4, contracted tail. (i) FWLLm5, non-contracted tail. (j) FWLLm5, contracted tail. (k) and (l) Lamda-like phage, presented in FWLLm2 stock. (m) A511, non-contracted tail. (n) A511, contracted tail (Scale represents 100nm).

L. monocytogenes phage, LM A511 phage, is another well studied phage which was provided by Fonterra and attached to LM 3009. A511 was used as a reference phage in the study and was prepared into high titre stocks and as a control to ensure that the methods used were effective. A511 form a small clear plaque on lawns of LM 3009. Judging from the electron microscopy, it belongs to the *Myoviridae* family and has an isometric head, rigid contractile tail and branch like tail fibrils from its base plate (Figure 3.9). Phage A511 prepared in this study had an average head diameter of 97.7 nm ± 5.3 (n=32), an average tail length of 209.6 nm ± 11.5 (n=4), and an average tail diameter of 8.4 nm ± 0.9 (n=25).The head of A511 supplied by Fonterra was slightly larger than the A511 phage reported by others (Zink and Loessrov, 1992) (i.e. 88 nm ± 2.4 vs. 97.7 nm ± 5.3).

3.6.2 Host Range

The host range of *Listeria* phages was assessed by spot test onto lawns of 12 *Listeria* species and strains including the indicator stains used to initially isolate the phage (Table 3.6). High titre stocks of phage were freshly prepared with the concentration of stocks measured and diluted to corresponding final titres of 10^2, 10^4, and 10^6 pfu/ml. FWLLm1, FWLLm2, FWLLm3, and FWLLm4 phages did not produce plaques on *L. grayi* and FWLLm1 also did not form plaques on *L. inoocu*. FWLLm3 and FWLLm4 did not form plaques on *L. ivanovii*. In contrast, FWLLm5 phage did form plaques on all 12 *Listeria* indicator hosts, indicating that it had a broader host range. Phage A511 form plaques on all lawns except those make with *L. monocytogenes* 2000/47.

Each *Listeria* phage had different plaque-forming efficiencies on attaching individual host species but all have a broad host ranges. For example, FWLLm3 and FWLLm4 obviously

reduced *L. monocytogenes* (NZRM 3370) growth on TSB base plates, but no distinct plaques were observed at higher titer stock (10^6 pfu/ml) that spotted on the lawn. The other 4 phages, FWLLm1, FWLLm2, FWLLm5 and A511, form plaques at lower titre (10^2 pfu/ml). This type of lysis was caused by infection of the bacteria by a single phage particle and multiplication of this phage particle up to a threshold value. The cell contents were then liberated into solution without deformation of the cell wall. Whereas FWLLm3 and FWLLm4 phages' ability to lysis *L. monocytogenes* (NZRM 3370) were caused by lysis from without, meaning that lysis caused by adsorption of phage above a threshold value of phage concentration. The cell contents were liberated by a "non-productive infection" of distension and destruction of the cell wall. Inhibition zones that formed at the highest titre of phages added and not at 1/10 or 1/100 dilutions of phage stocks may suggest them of lysis from without.

3.6.3 Lysis Ability of *Listeria* phages

L. monocytogenes lysis was monitored by OD readings at 660 nm and were used as an indicator of *L. monocytognenes* present in the broth and used to assist the characterisation of *Listeria* phages. The average growth of the bacteria was described as a sigmoid curve (n=3), in the absence of phage, but was interrupted by the addition of the FWLLm phages (Figure 3.10). Each phage's ability to lyse the host was also assessed at two different phage to host ratios.

Table 3.6 Host range of isolated *Listeria* phages and phage A511

Indicator hosts	**FWLLm1 (pfu/ml)**	**FWLLm2 (pfu/ml)**	**FWLLm3 (pfu/ml)**	**FWLLm4 (pfu/ml)**	**FWLLm5 (pfu/ml)**	**A511 (pfu/ml)**
L. inoocua	-	10^6**	10^6**	10^6**	10^6**	10^6+
L.ivanovii	10^2+	10^2+	-	-	10^6**	10^2+
L.weishimeri	10^4+	10^4+	10^6**	10^6**	10^4+	10^4+
L.grayi	-	-	-	-	10^6**	10^6+
LM (NZRM 44)	10^2+	10^2+	10^4+	10^4+	10^2+	10^2+
LM (NZRM 2592)	10^2+	10^2+	10^2+	10^2+	10^2+	10^2+
LM (NZRM 2594)	10^2+	10^2+	10^2+	10^2+	10^2+	10^2+
LM (NZRM 3370)	10^2+	10^2+	10^6**	10^6**	10^2+	10^2+
LM (NZRM 3449)	10^6**	10^6**	10^4*	10^4*	10^6**	10^4+
LM (NZRM 3450)	10^6**	10^6**	10^4*	10^4*	10^6**	10^4+
LM 3009	10^2+	10^2+	10^6**	10^6**	10^6+	10^6+
LM 2000/47	10^4+	10^4+	10^2+	10^2+	10^2+	-

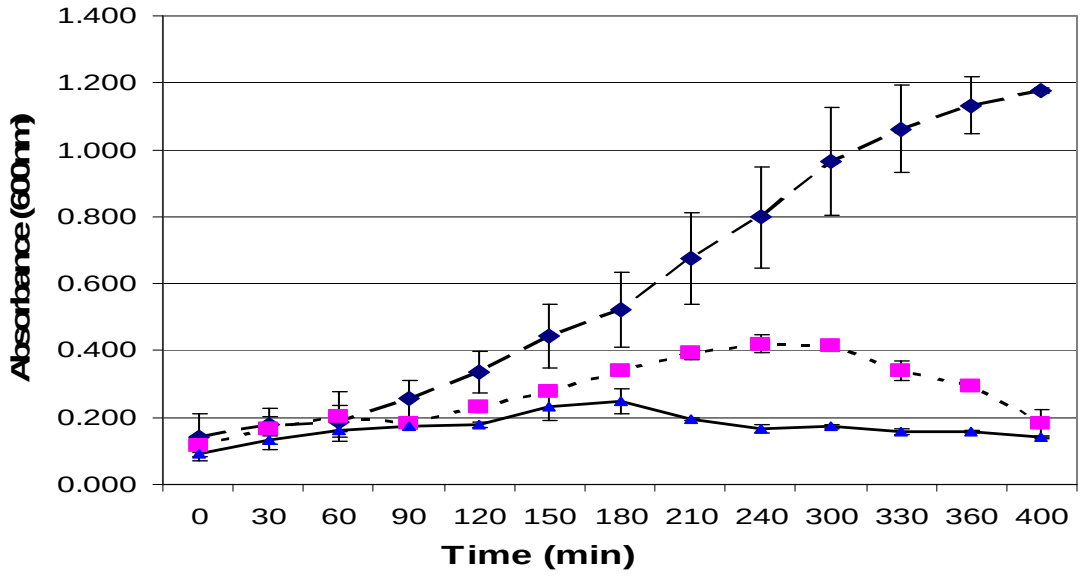
Starting titre for all FWLLm phages were 10^8 puf/ml, + distinct clear plaques, * host population obviously reduced but no distinct plaques ** likely to be lysis from without Lysis activity observed with highest phage concentration shown in brackets. LM refers to *L. monocytogenes.* Pfu numbers are determined by plating on each specified host that indicates an efficiency of plating measure.

The multiplicity of infection (MOI) was estimated by the initial phage concentration divided by the initial concentration of bacteria. In Figure 3.10 a, the MOI 1 and MOI 2 of FWLLm1 were calculated to be 0.98 and 1 respectively. With a ten-fold difference in the MOI, the broth with higher MOI shows lysis of the *Listeria* approximately 210 min after infection, while at lower MOI it took 400 min to lyse the *Listeria* host back to the original of OD reading (OD=0.118). The MOI of other phages were calculated to be similar to that of phage FWLLm1. However, the ability of each phage for lysis its host was different. For FWLLm2 and FWLLm3 (Figure 3.10 b and c), *Listeria* appeared to lyse 210 min and 30 min respectively after addition of phage stock at the higher MOIs, while with lower MOIs lysis took approximately 400 min. Phage FWLLm4 and FWLLm5 lysed *Listeria* 30 min and 300 min after addition of phage stock at high MOIs. However, phages FWLLm4 and FWLLm5 showed better ability at lysis the *Listeria* with lower MOI (240 min) then other phages.

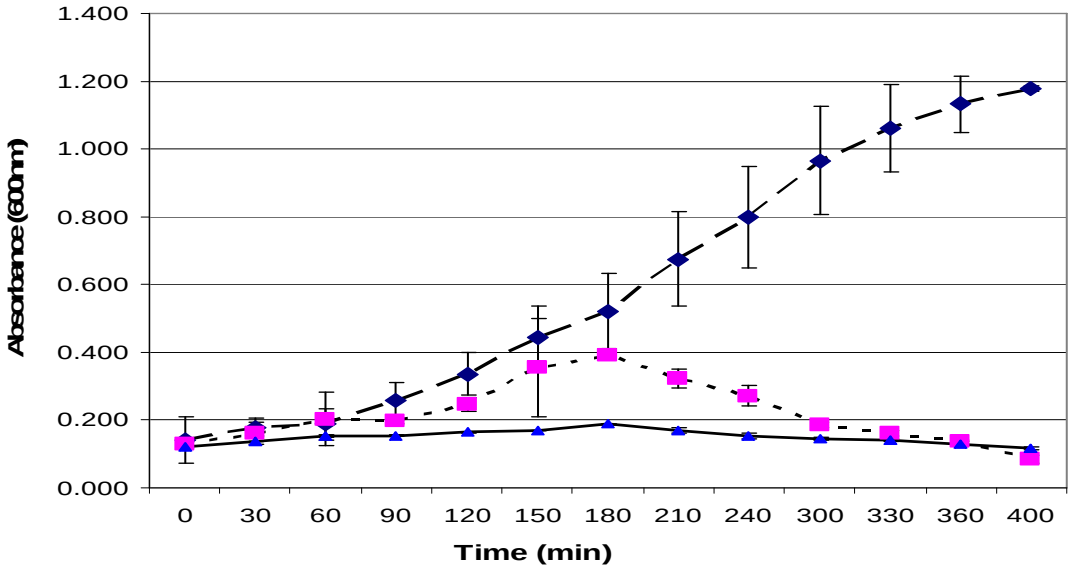
3.6.4 Stability During Frozen and Refrigerated Storage

The stability of phage under refrigerated and frozen conditions could assist with further characterization of the phage and help determine the best storage condition for future study. Phages FWLLm1, FWLLm2, FWLLm3, FWLLm4 and FWLLm5 were stored in LB broth, SM buffer, SM buffer+ gelatin (2%) and LB broth + 15 % glycerol at 22, 4 and -80°C. The stability of *L. monocytogenes* phages under different storage conditions are summarised in Table 3.7.

(a)


1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Absorbance (600nm)
0 30 60 90 120 150 180 210 240 300 330 360 400
Time (min)

(b)


1.400
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0.800
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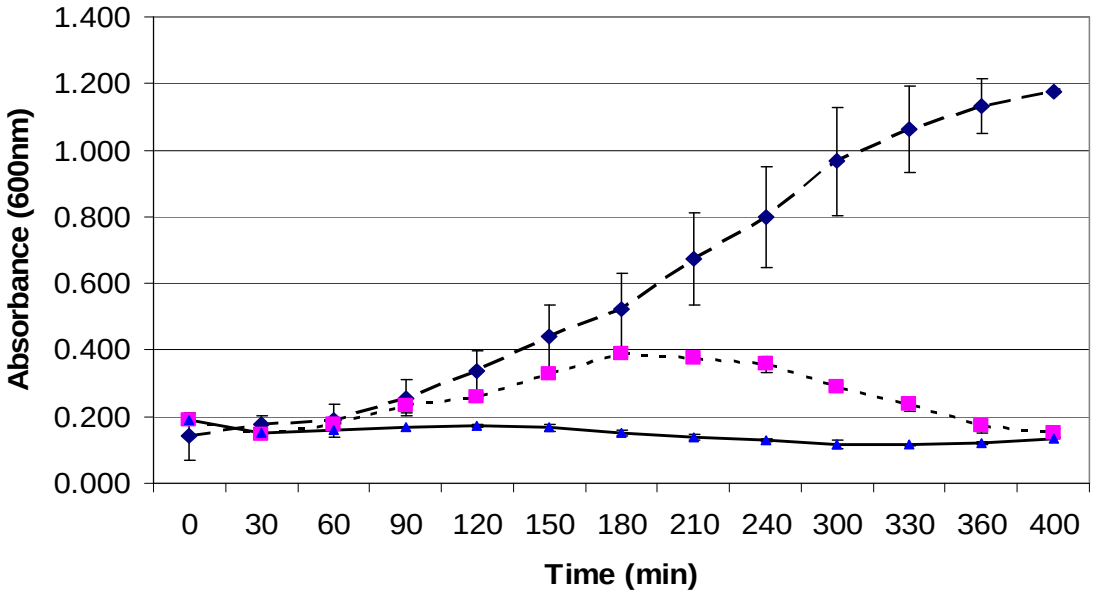
0.400
0.200
0.000
Absorbance (600nm)
0 30 60 90 120 150 180 210 240 300 330 360 400
Time (min)

(c)


1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Absorbance (600nm)
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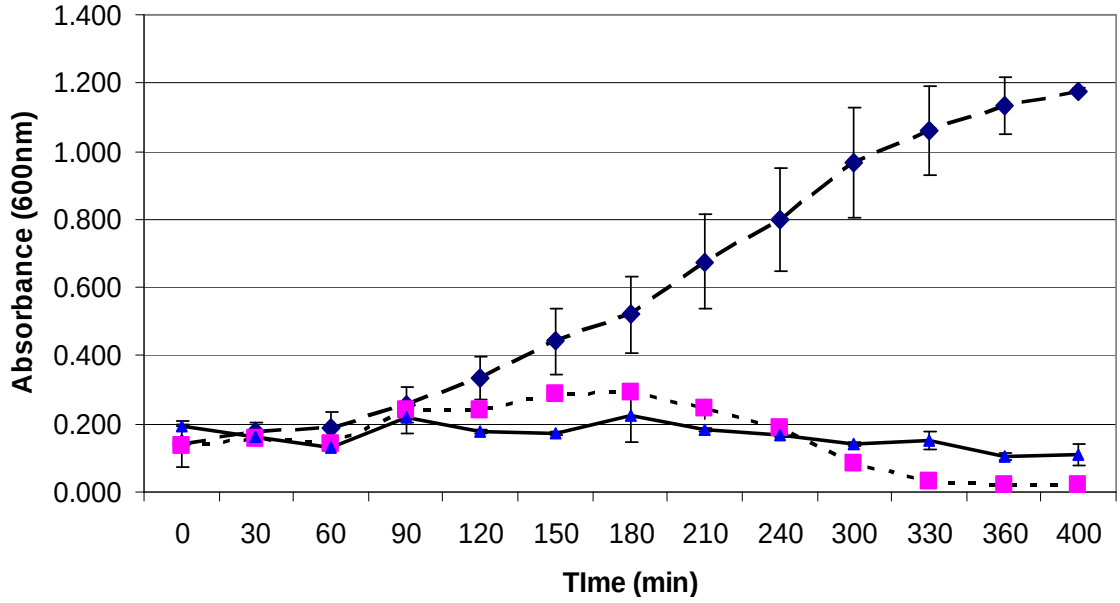
Time (min)

(d)



(e)

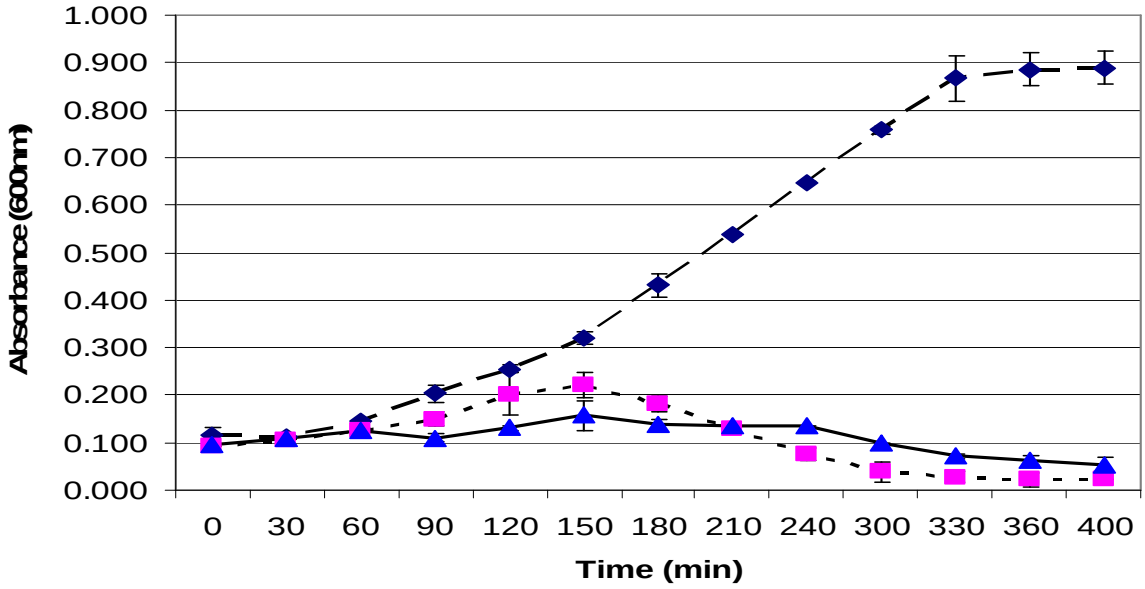


Figure 3.10 Lysis of *L. monocytogenes* (2000/47) by phages (a) FWLLm1, (b) FWLLm2, (c) FWLLm3, (d) FWLLm4 and (e) FWLLm5 on *L. monocytogenes* (3009) host. Dashed line represents *L. monocytogenes*, solid line represents *Listeria* host with phage to host ratio of 1, and the dotted line represents *Listeria* host phage to host ratio of 0.1. Phages were added to the host at time zero.

After 7 days of storage, a 10^3 pfu/ml reduction in phage concentration from the initial phage concentration was identified for FWLLm1 stored in SM + gelatin and LB broth at 4°C, with greater than 10^3 pfu/ml reductions recorded for stocks stored under other conditions. In fact, no plaques were observed in all the media stored at -80°C for FWLLm1 phage.

Phage FWLLm2 had a 10^2 pfu/ml reduction for 7 days of storage in LB broth and SM buffer + gelatin at 4°C. More than 10^3 pfu/ml reduction of phage concentration was noted for stocks stored in other conditions, and no plaques were observed from medium, SM buffer stored at -80°C after 7 days.

FWLLm3 phage remained at its initial phage concentration when stored in LB broth at 4°C for 7 days. A 10^1 pfu/ml reduction of phage concentration was noted in other conditions, while FWLLm3 stored in SM buffer, SM buffer + gelatin and LB broth + glycerol at -80°C reduced in concentration by 10^2 pfu/ml reduction. Phages FWLLm4 and FWLLm5 had a 10^1 pfu/ml reduction in phage concentration when stored in SM buffer + gelatin and LB broth respectively. Other storage conditions resulted in a 10^1 pfu/ml to 10^2 pfu/ml of reduction in phage concentration, and no plaques were observed from all media stored at -80°C after 7 days.

After 30 days of storage, FWLLm1 and FWLLm2 a 10^3 pfu/ml and 10^2 pfu/ml reduction respectively from their initial concentration when stored in LB broth and SM buffer + gelatin at 4°C. Under other storage conditions there were greater reductions from the initial phage concentrations. Phage FWLLm3 stayed at its initial concentration when stored in LB broth at 4°C, while FWLLm4 showed a 10^1 pfu/ml reduction from its initial concentration after 30 days of storage in SM buffer + gelatin at 4°C. Phage FWLLm5 was noted to have further 10^2

pfu/ml reductions in phage concentration from day 7 until day 30 stored in LB broth at 4°C. However, a 10^4 pfu/ml or more reduction from its initial phage concentration was noted for FWLLm5 stored under other conditions.

Table 3.7 Stability of FWLLm and A511 phages under various storage conditions.

		Day 7			Day 30		
Temperatures (°C)		**22**	**4**	**-80**	**22**	**4**	**-80**
LM1	**LB**	10^4	10^6	ND	10^2	10^4	ND
	LB+G	10^4	10^5	ND	10^3	10^3	ND
	SM+G	10^4	10^6	ND	10^3	10^3	ND
	SM	10^4	10^5	ND	10^2	10^2	ND
LM2	**LB**	10^2	10^4	ND	10^1	10^4	ND
	LB+G	10^2	10^3	ND	10^1	10^2	ND
	SM+G	10^2	10^4	ND	10^1	10^3	ND
	SM	10^3	10^3	ND	10^1	10^2	ND
LM3	**LB**	10^4	10^5	10^2	10^3	10^4	ND
	LB+G	10^3	10^4	10^2	10^3	10^2	ND
	SM+G	10^4	10^5	10^2	10^3	10^3	ND
	SM	10^3	10^4	ND	10^2	10^2	ND
LM4	**LB**	10^3	10^7	ND	10^3	10^7	ND
	LB+G	10^2	10^5	10^4	10^2	10^4	ND
	SM+G	10^2	10^5	10^4	10^1	10^5	ND
	SM	10^2	10^5	10^4	10^1	10^3	ND
LM5	**LB**	10^4	10^6	ND	10^2	10^5	ND
	LB+G	10^5	10^5	ND	10^4	10^4	ND
	SM+G	10^4	10^6	ND	10^4	10^6	ND
	SM	10^4	10^5	ND	10^3	10^4	ND
A511	**LB**	10^5	10^7	10^5	10^4	10^6	10^3
	LB+G	10^5	10^5	10^5	10^3	10^4	10^3
	SM+G	10^5	10^7	ND	10^4	10^6	ND
	SM	10^5	10^5	ND	10^4	10^4	ND

Starting titres for all phages were 10^7 pfu/ml, LM refers to *L. monocytogenes* and ND refers to titre of phage were under the limit of detection. LB refers to LB broth, LB+G refers to LB broth 15 % glycerol, SM+G refers to SM buffer + gelatin (2%), and SM refers to SM buffer.

In comparison, phage A511 remained its initial phage concentration when stored in either SM buffer or LB broth at 4°C after 7 days of storage. No plaques formed from a stock stored in SM buffer or SM buffer + gelatin at -80°C, and a 10^2 pfu/ml of reduction of phage concentration in other storage conditions. After 30 days of storage, phage A511 had a further 10^1 pfu/ml of reduction of its phage concentration in either SM buffer or LB broth at 4°C. While other storage conditions showed a total of 10^3 pfu/ml to 10^4 pfu/ml of phage reduction from its initial concentration for phage A511. Therefore, *L. monocytogenes* phages were stored in conditions shown in Table 3.8 for later use.

Table 3.8 Best storage conditions for *Listeria* phages after storage for 30 days

		Best storage conditions
L. monocytogenes phages	**FWLLm1**	LB broth or SM buffer + gelatin at 4°C
	FWLLm2	LB broth or SM buffer + gelatin at 4°C
	FWLLm3	LB broth at 4°C
	FWLLm4	SM buffer + gelatin at 4°C
	FWLLm5	LB broth at 4°C
	A511	LB broth or SM buffer at 4°C

3.6.5 Effect of Chloroform

The chloroform resistance of phage could add on to the knowledge of phage biochemistry and hence the characterization of phage. Fresh high titre stock of FWLLM phages were prepared with titre were measured. A drop of chloroform was applied to each titre stock of phage. All FWLLm phages, including A511, were able to show same phage titre after exposed to chloroform for 30min at 22°C. This indicated the present of chloroform had no effect on all *Listeria* phages isolated because most phages responded immediately (within few seconds)

(Alatossava and Klaenhammer, 1991; Oh et al., 1999) when chloroform was applied and therefore, is chloroform resistant.

Chapter 4

Discussion

4.1 Phage Isolation

Isolation of *Bacillus cereus* was relatively easy from mashed potato; however, *B. cereus* phages were not isolated from food samples (Section 3.2). This could be explained by that the *Bacillus* present in mashed potatoes was spores, so they survive better than phages would. Moreover, this is not to say that phages are not present in food. This observation may be a result of chance, experimental design, or of the limit of phage detection in this study. Consumers' confidence in the application of phages as a biocontrol agents on food may be more readily gained if the phages were shown to come from food. To achieve this, the methodology of recovering phage from the food samples will have to be improved, in terms of the rate of phage recovery, by performing tests on numerous food samples.

Two *B. cereus* phages were isolated from soil samples, consistent with the source of Thorne's isolation of *B. cereus* phage, named CP-51 (Thorne, 1968). The medium Thorne used (i.e. NBY) is different to media used in this thesis (i.e. BHI). However, both media are general-purpose nonselective media that are within the pH range (i.e. pH 6.8 to 7.5) preferred by *B. cereus*. Hence, the isolation procedure described here was not a significantly different from that used by Thorne.

B. cereus phages were tested against purified monocultures of strains and species, instead of using a 'cocktail' of hosts, another common method was used in the isolation of phages. By doing this, the progress of isolation might have been slower because the recovery rate of phage has decreased. Nevertheless, once phages were isolated, this method reveals instant

information about host range. The two *B. cereus* phages could not be compared with respect to specificity with CB-51 isolated by Thorne until further host range tests.

Unfortunately, two *B. thuringiensis* phages isolated from another soil sample (FWLBt1 and FWLBt2) were lost after storage at 4°C for a month in SM buffer. Returning to the same soil sample stored at 4°C to attempt to recover new phages was unsuccessful. This might due to the phage being unstable at 4°C regardless of medium. However, the actual reason for not finding new phage cannot be diagnosed with certainty.

The isolation of *Listeria monocytogenes* phages was more difficult than *B. cereus* phages. This may be because *Listeria* is relatively slow growing compared to *Bacillus*. In addition, the plaques on lawns of *L. monocytogenes* were only observed at 22°C even though *L. monocytogenes*' optimum growth temperature is 30°C. Possibly lytic *Listeria* phages are less active at the host's optimum laboratory growth temperature.

4.2 Electron Microscopy

Bacillus specific phages were distinguished morphologically using an electron microscope (Section 3.3.1). The concentration method used (Section 2.10.1) was successful in providing phages in good condition. In addition, the reference phage, A511, has measurements similar to literature reports and thus provides confidence in the concentration procedure used here. Although it should be noted that the dimensions of A511 reported in this study were larger than it is in the literature (Zink and Loessner, 1992), this difference was not statistically significant. Furthermore, since A511 is now laboratory adapted, there may be an unknown selection or drift affecting it as it is passed from investigator to investigator.

Both FWLBc1 and FWLBc2 had similar head, tail, sheath, and tail tube dimensions. The previously described *B. cereus* phage Bace-11 also had a similar head size to FWLBc phages isolated (Ackermann et al., 1995). However, the giant contractile tail (485 x 20 nm) of Bace-11 along with three long wavy tail fibers distinguished from the FWLBc phages (Ackermann et al., 1995). FWLBc phages looked similar to phage SP50 and Bastille (Eiserling and Boy de la Tour, 1965). Although there are also other phages of identical morphology, these two particular phages were chosen for comparison because they infect *B. cereus* (strain 14579 and HER1399 respectively) (Walter and Dalten, 2003; Loessner et al, 1997). Although FWLBc phages showed to be slightly bigger, statically, than SP50 and Bastille (Jarvis et al., 1993), the difference was not significant (i.e. between 2-7 nm), considering the standard error in measurements. Nevertheless, both FWLBc phages isolated in this study are classified into A1 morphology of the *Myoviridae* family.

L. monocytogenes phages were isolated from two ruminant faeces samples and distinguished by their plaque morphology. They were then further divided into three different types according to their host specificity and dimensions under the electron microscope. FWLLm1, FWLLm2 and FWLLm4, grouped as type one, were morphologically similar to *L. monocytogenese* A511 phage reported by Zink and Loessner (Zink and Loessner, 1992). These phages had larger features than those published by others, although the ratio of tail length to head diameter is comparable.

FWLLm3 and FWLLm5 were isolated from the same ruminant faecal sample and made similar looking plaques, and had bigger head diameter and shorter tail length than other FWLL phages. Their larger size was the inverse of their relative plaque sizes. Adam explained such observations previously by suggesting that larger phages diffuse less rapidly through the soft-agar overlay and therefore the site of infection is smaller (Adams, 1959). FWLLm3 and

FWLLm4 phages had similar dimensions and therefore a comparable head to tail ratio. Although all five FWLLm phages isolated in this study had the same morphology as A511 phage, they were distinguished by some differences in dimensions and their host ranges.

4.3 Host Range

While the host ranges of FWLBc1 is not identical to FWLBc2, the host range of the *B. cereus* phages may be generally described as narrow. FWLBc1 and FWLBc2 phages only lyse 36% and 27%, respectively, of the hosts tested. These numbers are derived by assuming that A2 and D2 are the same strain because they were identical by all tests conducted. It was noted that FWLBc2 had more lysis from without (LWO) activity. As the morphology of FWLBc phages was similar to SP 50 and Bastille phages, their host range specificity was compared here. SP 50 phage was reported to lyse *subtilis*, however, FWLBc phages did not. Nevertheless, SP 50 was tested on a different strain of B. subtilis (i.e. HWA 1243) (Walter and Baker, 2003) than strain used in this study. Bastille phage's ability to lyse both *B. cereus* and *B. thuringiensis* (Jarvis et al., 1993) suggested that FWLBc phages isolated in this study could be the same as Bastille and not the SP 50 phage reported earlier. The host range of FWLBc phages also suggests that they will not be useful biocontrol agents, at least in applications using them individually, because they did not lyse isolated *B. cereus* strains NCTC 11143, NCTC 11145 and NCRM5. However, more *Bacillus* species, especially *B. subtilis* and *B. thuringiensis*, and strains are needed for further host range tests to verify this.

The *L. monocytogens* FWLLm phages had a broad host range and lysed 50-75 % of the *L. monoctygens* strains tested. Typing phages A511 and P100 are known to be useful biocontrol agents because of their broad host ranges. The broad host range of FWLLm phages suggests that these phages could also be useful biocontol agents. Furthermore, all five FWLLm phages were able to infect *L. monocytogenes* 2000/47, a strain which recurs in New Zealand clinical

cases, while the reference phage A511 did not. This unique feature makes FWLLm phages of special interest especially in New Zealand. Almost all of the *Listiera* phages are temperate and have a very narrow host range (Loessner and Rees, 2005); therefore, FWLLm phages could be useful for applications such as biocontrol agents.

4.4 Single-Step Growth

Both FWLBc phages rapidly lysed host cultures *in vitro*. In single-step growth, the values determined for the number of cells infected by the phages and the number of cells killed in single-step growth experiment supports the conclusion that the FWLBc phages were efficient at cell lysis in the conditions tested. The latent period and burst size calculated provided useful information of attachment time and infection success at optimal temperatures. The burst sizes of FWLBc phages were between 300-322, and the latent periods were between 102-106 min. The burst sizes were much larger than reported for other *Bacillus* phages of *Myoviridae* family, including phage SP 50 (burst size of 53) (Jarvis et al., 1993). FWLBc phages' latent periods were relatively longer than other phages belonging to the *Myoviridae* family, but were comparable to *Serratia liquefaciens* phages ΦCP6-4 and ΦCP6-4 (Ashelford et al., 1999). Based on the work of others using phage with long latent periods and large burst sizes (Abedon, 1989; Wang et al., 1996; Stewart and Levin, 1984), FWLBc phages may provide an advantage for use as biocontrol agents when susceptible host bacteria are scarce.

Due to difficulties and time spent on isolation of *L. monocygognene* phages, only the lysis ability of *L. monocytogenes* phages was conducted in this study. Each FWLLm phage had slightly different effectiveness in respect to its lytic activity on the host. The bacteria population was inactivated rapidly to a low optical density *in vitro*, showing the lytic ability of these phages.

4.5 Restriction Fragment Profiles

Due to difficulties and time spent on isolation of *L. monocygognene* phages, only the restriction fragment profiles of *B. cereus* phages were conducted in this study. The method developed by ESR (S. Ismail, personal communication, 2007) was used and the DNA samples prepared for *B. cereus* phages were sufficiently pure for restriction digestion. Phages of the *Myoviridae* family are suggested to be especially difficult to digest, because instead of cytosine, the DNA contains glucosylated hydroxymethyl cystosine (O'Flynn et al., 2004). Nevertheless, based on the results in this study, both FWLBc phages had similar restriction fragment profiles with a calculated genome size of approximately 134 kb, which is within the phage DNA size range of 95-166 kb (Jarvis et al., 1993). These results may indicate that the two phages isolates are very similar in their genome size and may be also have similar DNA sequences.

FWLBc phages have a larger genome size than most *Myoviridae* phages. It is hard to know whether this is a biologically significant observation. However, of more than 5000 bacteriophages that have been isolated (Ackermann, 2006), 96% have double-stranded DNA genomes; and of 496 deposited in databases (NCBI Entrez Genome, 2008), only 33 have genomes as long as 130 Kb. Calaverie and co-workers revealed a significant under-sampling of long-genome bacteriophages with a statistical analysis study (Claverie et al., 2006). Thus, perhaps a significance of this study will be in its contributing the minority of large genome bacteriophage descriptions. Further sequence studies of FWLBc phages might add more understanding to the genomic composition of these phages.

4.6 Stability Under Frozen and Refrigerated Storage

From the results of experiments designed to test phage survival under various storage conditions, it became apparent that both the FWLBc and FWLLm phages were much more stable at 4°C than at room or freezing temperatures. This particular outstanding difference from CP-51 (i.e. more stable at room temperature than at 2 to 4°C) (Thorne and Holt, 1974) also contributes to the suggestion that these FWLBc phages are novel phages.

The losses of both FWLBc and FWLLm phages after 30 days of storage at -80°C suggested their sensitivity to freezing temperatures. As reported by Clark and Geary, large, complex and osmotic-shock-sensitive phages are generally more vulnerable than small, osmotic-shock-resistant phages in this condition (Clark and Geary, 1973). This observation suggests that these phages might be complex and osmotic-shock-sensitive.

4.7 Chloroform resistance

All *B. cereus* and *L. monocytogenes* phage isolates were chloroform resistant. It is therefore supposed that all FWLBc and FWLLm phages lacked lipid components because lipids are soluble in nonpolar solvents such as chloroform. Chloroform is the most common agent for determining lipid presence because the response of the phages seems to be independent of the concentration of chloroform used (Price and Van Rooyen, 2001).

4.8 Biocontrol on Food

In this study, both FWLBc phages were isolated from soil samples and did significantly reduce bacteria populations in mashed potatoes within 24 h at room temperature. This result was only observed when the ratio of phage and host was 1000 at a host concentration of 10^5 cfu/ml, but not when phage to host ratio was 1. This indicated that big ratio of phage and host is needed for FWLBc phages to serve as food biocontrol agents. Experiments were also done

at lower temperatures (i.e. 4 and 10 °C) with a phage to host ratio of 1. No evidence of *B. cereus* reductions was observed at low temperature with small phage to host ratio. Low number of *B. cereus* bacteria (i.e. 10^1 to 10^2 cfu/ml) in the broth with phage concentrations remained reasonably stable (i.e. 10^6-10^7 pfu/ml) was observed at the end of experiment. The lack of reduction of *Bacillus* host at low temperatures may be explained that FWLBc phages propagate only in growing bacteria cultures (Schrader et al., 1997), and this *B. cereus* isolate from mashed potatoes does not grow well at low temperatures. Further study of various FWLBc phage to host ratios in low temperatures are needed to confirm this hypothesis. The FWLLm phages' ability to reduce pathogen on food will be continued by other students in ESR while this thesis is on its progress.

Nevertheless, FWLBc's ability on significantly reducing bacteria population in mashed potato could contribute to an eventual "cocktail" of phages active against this species and perhaps other significant species of potentially pathogenic bacteria. Much work is required to assess all the potential interactions of phages and their hosts. Investigation on the phages' effect on changing the ecology before implementation as a biocontrol is also crucial.

Chapter 5

Conclusion

The findings in this study will add to the current knowledge of phages in the context of various environmental conditions for different bacteria and may draw us closer to discovering whether phages can be used to control bacterial contaminants of food. Isolation of phages able to infect Gram positive foodborne pathogens *Bacillus cereus* and *Listeria monocytogenes* could not be isolated from food, but were from soil and ruminants faecal material. Several methods of phage isolation from food and effluent were trialled for both foodborne pathogens; however, much improvement on the methodology is required to increase the efficiency of phage recovery.

All of the isolated phages were characterised as the first step in assessing their potential as biocontrol agents. The host range of the phages was tested against a range of different bacteria. The *B. cereus* phages had a narrow host range. While *L. monocytogenes* phages had a broad host range. All five FWLLm phages were also able to infect *L. monocytogenes* 2000/47, a strain which recurs in New Zealand clinical cases. These phages may have other medical benefits.

Both *B. cereus* and *L. monocytogenes* phages appeared to be of the *Myoviridae* family, judging from their physical characterisation in electron microscope. Single-step growth curves were used to ascertain the phages' replication characteristics at optimum growth temperatures. The burst size of *B. cereus* FWLBc phages were 322 and 300 pfu with latent period of 106 and 102 min. The long latent period and large burst size of FWLBc phages indicated the potential efficacy of phages applied for biocontrol. They may also produce

enough progeny to be useful under conditions where hosts are scarce. Both *Bacillus* and *Listeria* phages were choloroform resistant and had a better stability at 4°C than at room or freezing temperatures. *B. cereus* FWLBc phages had similar restriction genome profiles and the same genome size, indicating that they are possibly closely related. By pure speculation, the two *B. cereus* phages might be the mutant of one another due to their similarity in characterisations with difference only in plaque morphology and host range. Nevertheless, sequence study will need to confirm this speculation.

Phage activity in mashed potato was examined with *B. cereus* phages at variable temperatures. The bacteria population was significantly reduced in mashed potato within 24 h at room temperature using a high phage to host ratio. Although no *L. monocytognenes* FWLLm phage was tested on food, FWLLm phages did show promise because they rapidly reduced the host population. Much investigation is still required to implement this concept effectively and safely. Study on the foodborne bacteria would be important for the prediction of the likely effects of the biocontrol. Moreover, it is also important to establish all of the phage interactions with bacteria, such as whether the phage may increase the release of toxins from lysed bacteria and the potential for phage to transfer genes from one bacterium to another.

The application of phage to reduce the incidence of foodborne disease by inactivation of pathogens without affecting the quality of the food would be highly beneficial to the welfare of the health and economics of society. The potential of phages as biocontrol agents has been subject to numerous reviews (Adhya, 2003; Krylov, 2001; McKenna, 2001; Lorch, 1999) and several options to their application exist, but with applying phage directly onto the food are favoured (Atterbury et al., 2003; Leverentz et al., 2003; Carlton et al., 2005). The safety of phage therapy as biocontrol agents will undoubtedly face much greater examination prior to using it in food as biocontrol agent with the effect of concentrating phages in food and in

consumer and the effect of changing their niche in ecology must be investigated.

Chapter 5 Conclusion

References:

Abedon, S. T. (1989) Selection for bacteriophage latent period length by bacterial density: a theoretical examination. *Microbiology Ecology* **18**:79–88

Abedon, S. T. (ed) (2002) *The Bacteriophages*. Ed 2, Oxford University Press

Ackermann, H. W. (2003) Bacteriophage observations and evolution. *Research in Microbiology* **154**(4): 245-251

Ackermann, H. W. (2007) 5500 Phages examined in the electron microscope. *Archives of Virology* **152**:227-243

Ackermann, H. W. (ed) (2006) *Classification of bacteriophages.* In The bacteriophages. Oxford University Press

Ackermann, H. W., Audurier, A., and Rocourt, J. (1981) Morphologies de bactériophages de *Listeria monocytogenes*. *Annual Virology (Institut Pasteur)* **132E**:371-382

Ackermann, H. W., Roy, R., Martin, M., Murthy, M. R., and Smirnoff, W. A. (1978) Partial characterization of a cubic *Bacillus* phage. *Canadian Journal of Microbiology* **24**(8):986-993

Ackermann, H. W., Yoshino, S., and Ogata, S. (1995) A *Bacillus* phage that is a living fossil. *Canadian Journal of Microbiology* **41**:294-287

Adams, M. H. (1959) *Bacteriophages.* New York: Interscience Publishers, INC.

Adhya, S. (2003) Phage therapy, R. Calendar (ed) *The Bacteriophages. Oxford University Press*

Alatossava, T., and Claenhammer, T. R. (1991) Molecular Characterization of Three Small Isometric-Headed Bacteriophages which Vary in Their Sensitivity to the *Lactozoccal* Phage Resistance Plasmid pTR2030. *Applied and Environmental Microbiology* **57**(5):1346-1353

Alisky, J., Iczkowshi, K., Rapoport, A., and Troitsky, N. (1998) Bacteriophages Show Promises as Antimicrobial Agents. *Journal of Infection* **36**: 5-15

Alterbern, R. A., and Stull, H. B. (1964) Edema factor and phospholipase release by a strain of *Bacillus cereus*. *Canadian Journal of Microbiology* **10**: 717-725

Armon, R. K. (1996) Bacteriophages as Indicators of Pollution. *Critical Reviews in Environmental Science and Technology* **26**: 299-335

Ash, C., Farrow, J. A., Dorsch, M., Stackebrandt, E., and Collins, M. D. (1991) Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *International Journal of Systematic Bacteriology* **41**(3):343-346

Ashelford, K. E., Fry, J. C., Bailey, M. J., Jeffries, A. R., and Day, M. J. (1999) Characterization of Six Bacteriophages of *Serratia liquefaciens* CP6 Isolated from the Sugar Beet Phytosphere. *Applied and Environmental Microbiology* **65**(5):1959–1965

Atterbury, R, J., Connerton, P. L., Dodd, C. E. R., Rees, C. E. D., and Connerton, I. F. (2003b) Isolation and characterisation of *Campylobacter* bacteriophages from retail poultry. *Applied and Environmental Microbiology* **69**: 4511-4518

Atterbury, R. J., Connerton, P. L., Dodd, C. E. R., Rees, C. E. D., and Connerton, I. F. (2003) Application of Host-Specific Bacteriophages to the Surface of Chicken Skin Leads to a Reduction in Recovery of *Campylobacter jejunii*. *Applied and Environmental Microbiology* **69**(10): 6302–6306

Audurier, A., Rocourt, J., and Courtieu, A. L. (1977) Isolement et charactérisation de bactériophages de *Listeria monocytogenes*. *Annales de Microbiologie (Institut Pasteur)* **128A**: 185-188

Avery, S. M., Hudson, J. A., and Buncic, S. (1996) Multilocus enzyme electrophoresis typing of New Zealand *Listeria monocytogenes* isolates. *International Journal of Food Microbiology* **28**: 351-359

Barrow, P. A., and Soothill, J. S. (1997) Bacteriophage therapy and prophylaxis: rediscovery and renewed interest of potential. *Trends Microbiology* **5**:268-271

Bennion, M., and Scheule, B. (2004) *Introductory foods.* Ed 12. Upper Saddled River, NJ. Pearson Education International

Bergh, O., Borsheim, K. Y., Brabak, G., and Heldal, M. (1989) High abundance of viruses found in aquatic environments. *Nature* **340**:467-468

Beuchat, L. R., Clavero, M. R., and Jaquette, C. B. (1997) Effects of nisin and temperature on survival, growth, and enterotoxin production characteristics of psychrotrophic *Bacillus cereus* in beef gravy. *Applied Environmental Microbiology* **63(**5): 1953–1958

Biebricher, C. K., and Gardiner, W. C. (1997) Molecular evolution of RNA in vitro. *Biophysical Chemistry* **66**: 179-192

Bigwood, T., Hudson, J. A., Billington, C., Carey-Smith, G. V., and Heinemann, J. A. (2008) Phage inactivation of foodborne pathogens on cooked and raw meat. *Food Microbiology* **25(**2): 400-406

Billington, C. (2008) Environmental Science and Research (ESR), Christchurch Science Centre, personal communication

Boxall, N., and Ortega, J. (2003) Annual summary of outbreaks in New Zealand in 2002. ES*R Clint Report FW0151. Porirua: ESR*

Bradley, D. E. (1967) Ultrastructure of bacteriophages and bacteriocins. *Bacteriology Reviews* **31**:230-314

Brett, M.S.Y., Short, P., and McLauchlin, J. (1998) A small outbreak of listeriosis associated with smoked mussels. *International Journal of Food Microbiology* **43**: 223–229

Bruttin, A., and Brussow, H. (2005) Human volunteers receiving *Escherichia* coliphage T4 orally: a safety test of phage therapy. *Antimicrobial Agents Chemotherapy* **49**:2874

Burdon, K. L. (1946) Fatty material in bacteria and fungi revealed by staining dried, fixed slide preparations. *Journal of Bacteriology* **52**:665-678

Carey-Smith, G. V. (2004) *The use of bacteriophages as a biocontrol mechanism for Campylobacter and Salmonella contaminant of food.* Master of Environmental Science, University of Canterbury, New Zealand

Carey-Smith, G. V., Billington, C., Hudson, J. A., and Heinemann, J. A. (2006) Isolation and characterisation of bacteriophages infecting *Salmonella* spp. *FEMS Microbiology Letters* **258**: 182-186

Carlson, K., and Miller, E. S. (1994) Experiments in T4 Genetics. In *Molecular Biology of Bacteriophage T4*. Karam, J. D (ed) *Washington DC:* American Society for Microbiology

Carlton, R. M. (1999) Phage Therapy: Past History and Future Prospects. *Archivum Immunologiae et Therapiae Experimentalis* **47**:267–274

Carlton, R. M., Noordman, W. H., Biswas, B., de Meester E. D., and Loessner, M. J. (2005) Bacteriophage P100 for control of *Listeria monocytogenes* in foods: Genome sequence, bioinformatic analyses, oral toxicity study, and application. *Regulatory Toxicology and Pharmacology* **43**(3): 301-312

Cheun, H. I., Makino, S. I., Watarai, M., Shirahata, T., Uchida, I., and Takeshi, K. (2001) A simple and sensitive detection system for *Bacillus anthracis* in meat and tissue. *Journal of Applied Microbiology* **91**(3): 421–426

Clark, W. A., and Geary, D. (1973) Proceeding: Preservation of bacteriophages by freezing and freeze-drying. *Cryobiology* **10**:351-360

Claverie, J. M., Ogata, H., Audic, S., Abergel, C., Suhre, K., and Fournier, P. E. (2006) Mimivirus and the emerging concept of "giant" virus. *Virus Research* **117**:133–144

Coliner, A. R. (1948) The action of *Bacillus cereus* and related species on the lecithin complex of egg yolk. *Journal of Bacteriology* **55**:777-785

Connerton, P. L., Loc Carrillio, C. M., Swift, C., Dillon, E., Scoot, A., and Reed, C. E. D. (2004) Longitudinal Study of *Campylobacter jejuni* Bacteriophages and Their Hosts from Broiler Chickens. *Applied and Environmental Microbiology* **70**:3877-3883

Cooney, P. H., Jacob, R. J., and Slepecky, R. A. (1975) Characteristics of a *Bacillus megaterium* Bacteriophage. *Journal of General Virology* **26**:131-134

D'Ari, R. (1985) The SOS system. *Biochimie* **67**: 343-347

d'Herelle, F. C. R. (1917) Sur un microbe invisible antagoniste des bacilles dysentériques. *Academy Science Ser.* **165**: 373

Daeschel, M. A. (1993) Applications and interactions of bacteriocins from lactic acid bacteria in foods and beverages. In: Hoover, D. B., and Steenson, L. R. (eds) *Bacteriocins of Lactic Acid Bacteria.* New York: Academic Press INC. 63-91

Delbrück, M., and Ellis, E. L. (1939) The growth of bacteriophage. *The Journal of General Physiology* **22**(3): 365-384

Donovan, K. O. (1958). A selective medium for *Bacillus cereus* in milk. *Journal of Applied Bacteriology* **21**:100

Downes, F. P. and Ito, K. (eds) (2001) *Compendium of Methods for the Microbiological Examination of Foods:* American Public Health Association

Dufrenne, J., Bijwaard, M., te Giffel, M., Beumer, R., and Notermans, S. (1995) Characteristics of some psychrotrophic *Bacillus cereus* isolates. *International Journal of Food Microbiology* **27**: 175-183

Dykes, G. A., and Moorhead, S. M. (2002) Combined antimicrobial effect of nisin and a listeriophage against *Listeria monocytogenes* in broth but not in buffer or on raw beef. *International Journal of Food Microbiology* **73**: 71-81

Easingwood, R. (2008) Electron Microscopy Unit at the University of Otago, personal communication

EBI Food Safty. (2006b) Bacteriophages: The natural way to control food pathogens. URL: www.ebifoodsafety.com/en/profile-mission.aspx..

Eiserling, F. A., and Boy de la Tour, E. (1965) Capsomeres and other structures observed on some bacteriophages. *Pathology and Microbiology* **28**:175–180

ESR. (1998) Annual surveillance summary 1997. *Porirua: ESR*

ESR. (2007) National surveillance data. *NZ public health surveillance Rep. 5, 3*

Estela, L. A., Sofos, J. N., and Flores, B. B. (1992) Bacteriophage typing of *Listeria monocytogenes* cultures isolated from seafoods. *Journal of Food Protection* **55**: 13-17

Farber, J. M., and Peterkin, P. I. (1991) *Listeria monocytogenes*, a food-borne pathogen. *Microbiology of Molecular Biology Reviews* **55**(3): 476-511

Fauquet, C. M., Mayo, M. A., Maniloff, J., Desselberger, U., and Ball, L.A. (eds) (2005) *Virus Taxonomy: 8th Reports of the International Committee on Taxonomy of Viruses.* Elsevier. Academic Press, 1162

Favrin, S. J., Jassim, S. A., and Griffiths, M. W. (2003) Application of a novel immunomagnetic separation–bacteriophage assay for the detection of *Salmonella enteritidis* and *Escherichia coli* O157:H7 in food. *International Journal of Food Microbiology* **85**: 63-71

Flint, S. (2007) Fonterra Cooperative Ltd. Palmerston North, personal communication

Fraser, J. A., and Sperber, W. H. (1988) ISO 11290-1 Microbiology of food and animal feeding stuffs-Horizontal method for the detection and enumeration of *Listeria monocytogenes* Part 1 Detection method. *Journal of Food Protection* **51**(10): 762-765

González-Huici, V., Salas, M., and Hermoso, J. M. (2004) The push–pull mechanism of bacteriophage Ø29 DNA injection. *Molecular Microbiology* **52**(2):529–540

Goode, D., Allen, V. M., and Barrow, P. A. (2003) Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages. *Applied Environmental Microbiology* **69**: 5032-5036

Goodridge, L., and Abedon, S. T. (2003) Bacteriophage biocontrol and bioprocessing: Application of phage therapy to industry. *Feature Article* **53**(6): 254-262

Goodridge, L., Gallaccio, A., and Griffiths, M. W. (2003) Morphological, Host Range, and Genetic Characterization of Two Coliphages. *Applied Environmental Microbiology* **69**: 5364-5371

Goossens, H., Ferech, M., Stichele, R. V., and Elseviers, M. (2005) Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet* **365**:579-587

Hancock, R. W., and Braun, V. (1975) Nature of the energy requirement for the irreversible adsorption of bacteriophages T1 and phi80 to Escherichia coli. *Journal of Bacteriology* **125**(2): 409–415

Hofstra, H., van der Vossen, J. M. B. M., and van der Plas, J. (1994) Microbes in food processing technology. *FEMS Microbiology Reviews* **15**:175-183

Holbrook, R., and Anderson J. M. (1980) An improved selective and diagnostic medium for the isolation and enumeration of *Bacillus cereus* in foods. *Canadian Journal of Microbiology* **26** (7):753-759

Hudson, J. A. (2007) Environmental Science and Research (ESR) Christchurch Science Centre, personal communication

Hudson, J. A., Billington, C., Carey-Smith, G., and Greening, G. (2005) Bacteriophages as Biocontrol Agents in Food. *Journal of Food Protection* **68**(2): 426-437

Huss, H. H., Ababouch, L., and Gram, L. (2004) Assessment and management of seafood safety and quality. *Food and Agriculture organization of the United Nations. Rome*

ICMSF (International Commission on Microbiological Specifications for Foods) (1996) *Microorganisms in Foods 5. Characteristics of Microbial Pathogen,* Blackie Academic & Professional London

Inal, J. M. (2003) Phage Therapy: a Reappraisal of Bacteriophages as Antibiotics. *Archivum Immunologiae et Therapiae Experimentalis* **51**:237–244

Intralytix, INC. (2006c). Food Safety. URL www.intralytix.com/Intral_Food.htm.

Ismail, S. (2007) Environmental Science and Research (ESR) Christchurch Science Centre, personal communication.

Jarvis, A. W., Collins, L. J., and Ackermann, H. W. (1993) A study of five bacteriophages of the *Myoviridae* family which replicate on different Gram-positive bacteria. *Archives of Virology* **133**: 75-84

Kadavy, D. R., Shaffer, J. J., Lott, S. E., Wolf, T. A., Bolton, C. E., and Gallimore, W. H. (2000) Influence of infected cell growth state on bacteriophage reactivation levels. *Applied Environmental Microbiology* **66**: 5206-5212

Kennedy, J. E., and Bitton, G. (1987) Bacteriophages in foods, In: Goyal, S. M., Gerba, C. P., and Bitton, G. (eds) *Phage ecology.* John Wiley and Sons, New York 289-316

Krylov, B. (2001) Phage Therapy in Terms of Bacteriophage Genetics: Hopes, Prospects, Safety, Limitations. *Russian Journal of Genetics* **37**: 715-730

Kumar, V., Balaji, S., Gomathi, N. S., Venkatesan, P., Sekar, G., Jayasankar, K., and Narayanan, P. R. (2007) Phage cocktail to control the exponential growth of normal flora in processed sputum specimens grown overnight in liquid medium for rapid TB diagnosis. *Journal of Microbiological Methods* **68**: 536–542

Kwang, K., Frank, Y., and Joseph, F. (1994) Effect of Growth Nutrients on Attachment of *Listeria monocytogenes* To Stainless Steel. *Journal of Food Protection* **57**(8):720-724

Lake, R. J., Baker, M. G., Garrett, N., Scott, W. G., and Scott. H. M (2000) Estimated number of cases of foodborne infectious disease in New Zealand. *The New Zealand Medical Journal* **113**(1113):278-281

Lake, R., Hudson, A., and Cressey, P. (2004) Risk Profile: *Bacillus* spp. in rice. *Institute of Environmental Science and Research. Christchuch: ESR*

Leverentz, B., Conway, W. S., Alavidze, Z., Janiseiwicz, W. J., Fuches, Y., Camp, M. J., Chighladze, E., and Sulakvelidze, A. (2001) Examination of bacteriophage as a biocontrol method for *Salmonella* on fresh-cut fruit: a model study. *Journal of Food Protection* **64**:1116-1121

Leverentz, B., Conway, W. S., Camp, M. J., Janisiewicz, W. J., Abuladze, T., Yang, M., Saftner, R., and Sulakvelidze, A. (2003) Biocontrol of *Listeria monocytogenes* on Fresh-Cut Produce by Treatment with Lytic Bacteriophages and a Bacteriocin. *Applied and Environmental Microbiology* **69**(8): 4519-4526

Leverentz, B., Conway, W. S., Janisiewicz, W., and Camp, M. J. (2004) Optimizing Concentration and Timing of a Phage Spray Application To Reduce *Listeria monocytogenes* on Honeydew Melon Tissue. *Journal of Food Protection* **67**(8): 1682-1686

Levin, B. R., and Bull, J. J. (1996) Phage therapy revisited: the population biology of a bacterial infection and its treatment with bacteriophage and antibiotics. *The American Naturalist* **147**: 881-898

Liu, U., Dehbi, M., Moeck, G., Arhin, F., Bauda, P., Bergeron, D., Callejo, M., Ferretti, V., Ha, N., and Kwan, T. (2004) Antimicrobial drug discovery through bacteriophage genomics. *Natural Biotechnology* **22**:185-191

Loessner, M. J. (1991) Improved procedure for bacteriophage typing of *Listeria* stains and evaluation of new phages. *Applied and Environmental Microbiology* **57**: 882-884

Loessner, M. J. (1991) *Listeria Bakteriophaen: Isolierung, Charakterisierung und Anwendung in der Phagentypisierung.* Technische Universität München, Weihenstphan, Germany

Loessner, M. J. and Rees, C. E. D. (2005) *Listeria* phages: basics and applications. In: Waldor, M. K., Friedmann, D. I., Adhya, S. L. (eds) *Phage Their Role in Bacterial Pathogenesis and Biotechnology.* America Society for Microbiology, Washington, DC

Loessner, M. J., Krause, I. B., Henle, T., and Scherer, S., (1994) Structural proteins and DNA characteristics of 14 *Listeria* typing bacteriophages. Journal of General Virology **75**: 701-710

Loessner, M. J., Maier, S. K., Daubek-Puza, H., Wendlinger, G., and Scherer, S. (1997) Three *Bacillus cereus* bacteriophage endolysins are unrelated but reveal high homology to cell wall hydrolases from different Bacilli. *Journal of Bacteriophage* **179**(9): 2845-2851

Loessner, M., and Busse, M. (1990) Bacteriophage Typing of *Listeria* Species. *Applied and Environmental Microbiology*. **56**(6):1912-1918

Lopez, L., Baker, M., and Kieft, C. (2001) Annual summary of outbreaks in New Zealand in 2000. *ESR Clint Report FW0151. Porirua: ESR*

Lorch, A. (1999) Bacteriophages: An alternative to antibiotics? *Biotechnology and Development Monitor* **39**:14-17

Luria, S. E., and Human, M. L. (1952) A nonhereditary, host-induced variation of bacterial viruses. *Journal of Bacteriology* **64**(4): 557-569

MacKenzie, A. A., Perez, E., and Hathaway, S. (2004) Food systems and the changing patterns of foodborne zoonoses. *Scientific and Technical Review of the Office International des Epizooties* **23**(2): 677-684

Marks, T., and Sharp, R. (2000) Bacteriophages and biotechnology: a review *Journal of Chemical Technology and Biotechnology* **76**: 6-17

Marquet-Van Der Mee, N., and Audurier, A. (1995) Proposals for Optimization of the International Phage Typing System for *Listeria monocytogenes*: Combined Analysis of Phage Lytic Spectrum and Bariability of Typing Results. *Applied and Environmental Microbiology* **61**(1): 303-309

Marvin, D. A. (1998) Filamentous phage structure, infection and assembly. *Current Opinion in Structural Biology* **8(2)**:150-158

McGrath, S., and van Sinderen, D. (eds) (2007) *Bacteriophage: Genetics and Molecular Biology*, 1st ed., Caister Academic Press

McKenna, F., El-Tarabily, K. A., Hardy, G. E. S., T. and Dell, B. (2001) Novel in vivo use of a polyvalent *Streptomyces* phage to disinfest *Streptomyces* scabies-infected seed potatoes. *Plant Pathology* **50**:666-675

Modi, R., Hirvi, Y., Hill, A., and Griffiths, M.W. (2001) Effect of phage on survival of *Salmonella enteritidis* during manufacture and storage of cheddar cheese made from raw and pasteurized milk. *Journal of Food Protection* **64**: 927-933

Molineux, I. J. (2001) Micro Review: No syringes please, ejection of phage T7 DNA from the viron is enzyme driven. *Molecular Microbiology* **40**: 1-8

Mossel, D. A. A., Koopman, M. J., and Jongerius, E. (1967) Enumeration of *Bacillus cereus* in Foods. *Journal of Applied Microbiology* **15**(3):650-653

Meredith, L., Lewis, R., and Haslum, M. (2001) Contributory factors to the spread of contamination in a model kitchen. *British Food Journal* **103**(1):23-35

Mizoguchi, K., Morita, M., Fischer, C. R., Yoichi, M., Tanji, Y., and Unno, H. (2003) Coevolution of bacertiophage PP01 and *Escherichia coli* O157:H7 in continuous culture. *Applied and Environmental Microbiology* **69**:170-176

Murray, P., Rosenthal, K., and Kobayashi, G. (1998) Medical Microbiology. Ed 3. *Mosby*

Naing, T., Baker, M., Galloway, Y., and Margolin, T. (1999) Annual summary of outbreaks in New Zealand in 1998. *Institute of Environmental Science and Research. Porirua: ESR*

NCBI (2008) Entrez Genome URL www.ncbi.nlm.nih.gov/genomes/static/phg.html

Nissen, H., Rosnes, J. T., Brendehaug, J., and Kleiberg, G. H. (2002) Safety evaluation of sous vide-processed ready meals. *Letters in Applied Microbiology* **35**(5): 433-438

O'Flynn, G., Ross, R. P., Fitzgerald, G. F., and Coffey, A. (2004) Evaluation of a Cocktail of Three Bacteriophages for Biocontrol of *Escherichia coli* 0157:H7. *Applied and Environmental Microbiology* **70**: 3417-3424

Oh, J. S., Davies, D. R., Lawson, J. D., Arnold, G. E., and Dunker, A. K. (1999) Isolation of Chloroform-resistant Mutants of Filamentons Phage: Localization in Models of Phage Structure. *Journal of Molecular Biology* **287**: 449-457

Omnilytics. (2006) What makes bacteriophage safe? URL www.omnilytics.com/documents/What%20Makes%20Bacteriophage%20Safe.pdf.

Ortel, S., and Ackermann, H. W. (1985) Morphologie von neuen Listeriaphagen. *Zentralblatt für Bakteriologies und Hygiene, Abteilung 1 (Originale, Reihe A)* **260**:423-427

Pearson, L. J., and Marth, E. H. (1990) *Listeria monocytogenes* – Threat to a Safe Food Supply: A Review. *Journal of Dairy Science* **73**:912-928

Perks, M., Baker, M., and Galloway, Y. (2000) Annual summary of outbreaks in New Zealand in 1999. *Institute of Environmental Science and Research. Porirua: ESR*

Petran, R. L., and Swanson, K. M. J. (1993) Simultaneous growth of *Listeria monocytogenes* and *Listeria innocua*. *Journal of food protection* **56**(7): 616-618

Petty, N. K., Evan, T. J., Fineran, P. C., and Salmond, G. P. (2007) Biotechnological exploitation of bacteriophage research. *Trends in Biotechnology* **25**:7-15

Price, C., and Van Rooyen, J. (2001) Characterisation and identification of isolated bacteriophage from a polluted water source. *Journal of Molecular & Cell Biology* **177**(1): 44–151

Prescoot, A. G. (1993) A dilemma of dioxygenases (or where biochemistry and molecular biology fail to meet). *Journal of Experimental Botany* **44**: 849-861

Purves, W. K., Orians, G. G., and Heller, H. C. (1995) Life: The Science of Biology. *Massachusetts: Sinauer Associates, INC.*

Radding, C. M., Rosenzweig, J., Richards, F., and Cassuto, E. (1971) Separation and characterization of exonuclease, β protein, and a complex of both. *Journal of Biological Chemistry* **246**: 2510-2512

Randerson, J. (2003) Virus Cleans up Food Poisoning Bug. *New Scientist* 2392

Reacher, M. H., Shah, A., Livermore, D. M., Wale, C. J., Graham, C., Johnson, A. P., Heine, H., Monnickendam, M. A., Barker, K. F., James, D., and George, R. C. (2000) Bacteraemia and antibiotic resistance of its pathogens reported in England and Wales between 1990 and 1998: trend analysis. *British Medical Journal* **320**(7229): 213-216

Rhodehamel, E. J., and Harmon, S. M. (1998) *Bacillus cereus*, U.S. Food& Drug Administration, Center for Food Safety & Applied Nutrition.

Rhodemhamel, E. J., and Harmon, S. M. (eds) (1998) *Bacteriological Analytical Manual*, Ed 8, Revision A

Ripp, S., and Miller, R. V. (1998) Dynamics of the pseudo lysogenic response of slowly growing cells of *Pseudomonas aeruginosa*. *Microbiology* **144**: 2225-2232

Rocourt, J., Ackermann, H. W., Martin, M., Schrettenbrunner, A., and Seeliger, H. P. R. (1983) Morphology of *Listeria innocua* bacteriophages. *Annual Virology (Institut Pasteur)* **134E**: 245-250

Rocourt, J., Schrettenbrunner, A., and Seeliger, H. P. R. (1982) Isolation of bacteriophages from *Listeria monocytogenes* serovar 5 and L. innocua. *Zentralblatt für Bakteriologies und Hygiene, Abteilung 1 (Originale, Reihe A)* **251**: 505-511

Romig, W. R., and Brodetsky, A. M. (1961) Isolation and Preliminary Characterization of Bacteriophages for *Bacillus subtilis*. *Journal of Bacteriology* **82**(1): 135–141

Said, A. A., Iwabuchi, N., Matsui, T., Hirota, K, Kidokoro, S., Arai, M., Kuwajima, K., Schuck, P., and Arisaka, F. (2003) Reversible and Fast Association Equilibria of a Molecular Chaperone, gp57A, of Bacteriophage T4. *Biophysical Journal*. **85**:2606-2618

Schrader, H. S., Schrader, J. O., Walker, J. J., Wolf, T. A., Nickerson, K. W., and Kokjohn, T. A. (1997) Bacteriophage infection and mulitiplication occur in Pseudomonas aeruginosa starved for 5 years. *Canadian Journal of Microbiology* **43**: 1157-1163

Schuchat, A., Swaminathan, B., and Broome, C. V. (1991) Epidemiology of human listeriosis. *Clinical Microbiology Reviews* **4**:169-83.

Schultz, E.W. (1945) Listerella infections: a review. *Stanford Med Bull* **3**:135– 151

Scott, W. G., Scott, H. M., Lake, R. J., and Baker, M. G. (2000) Economic cost to New Zealand of foodborne infectious disease. *The New Zealand Medical Journal* **113**(1113):281-284

Seeley, N. D., and Primrose, S. B. (1982) The isolation of bacteriophages from the environment. *Journal of Applied Bacteriology* **53**(1):1-17

Seeliger, H. P. R. (1961) Listeriosis. *Hafner Publishing Company, Inc., New York, NY.*

Serwer P., Hayes, S. J., Zaman, S., Lieman, K., Rolando, M., and Hardies, S. C. (2004) Improved isolation of undersampled bacteriophages: finding of distant terminase genes. *Virology* **329**:414-424

Serwer, P. Hayes, S. J., Zaman, S., Lieman, K., Rolando, M., and Hardies, S. C. (2004) Propagating the missing bacteriophages: a large bacteriophage in a new class. *Virology* **329:**412-424

Sharp, R. (2001) Bacteriophages: Biology and History. *Journal of Chemical Technology and Biotechnology* **76**: 667-672

Sim, J., Hood, D., Finne, L., Wilson, M., Graham, C., and Hudson, J.A. (2002) Series of incidents of *Listeria monocytogenes* non-invasive febrile gastroenteritis involving ready-to-eat meats. *Letters in Applied Microbiology* **35**(5): 409-413

Skurnik, M., and Strauch, E. (2006) Phage therapy: facts and fiction. *International Journal of Medical Microbiology* **296**:5-14

Smith, H. W., and Huggins, M. B. (1980) The association of the O18, K1, and H7 antigens and the Col V plasmid of a strain of *Escherichia coli* with its virulence and immunogenicty. *Journal General Microbiology* **121**: 387-400

Smith, H. W., and Huggins, M. B. (1982) Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. *Journal General Microbiology* **128**: 307-318

Smith, H. W., and Huggins, M. B. (1983) Effectiveness of phages in treating experimental *E. coli* diarrhoea in calves, piglets, and lambs. *Journal General Microbiology* **129**: 2659-2675

Smith, H. W., Huggins, M. B., and Shaw, K. M. (1987) Factors influencing the survival and multiplication of bacteriophages in calves and in their environment. *Journal General Microbiology* **133**: 1127-1135

Smith, H. W., Huggins, M. B., and Shaw, K. M. (1987) The control of experimental *E. coli* diarrhoea in calves by means of bacteriophages. *Journal General Microbiology* **133**: 1111-1126

Stewart, F. M., and Levin, B. R. (1984) The population biology of bacterial viruses: why be temperate. *Theoretical Population Biology* **26**:93–117

Strauch, M. A., Spiegelman, G. B., Perego, M., Johnson, W. C., Burbulys, D., and Hoch, J. A. (1989) The transition state transcription regulator abrB of *Bacillus subtilis* is a DNA binding protein. *The EMBO Journal* **8**(5): 1615–1621

Sulakvelidze, A., Alavidze, Z., and Morris, J. G. J. (2001) Bacteriophage therapy. *Antimicrobial Agents Chemotherapy* **45**: 649-659

Thiel, K. (2004) Old dogma, new tricks – 21st Century phage therapy. *Nature Biotechnology* **2**(1): 31-36

Thorne, C. B. (1968) Transducing Bacteriophage for *Bacillus cereus*. *Journal of Virology* **2**(7): 657-662

Thorne, C. B., and Holt, S. C. (1974) Cold Lability of *Bacillus cereus* Bacteriophage CP-51. *Journal of Virology* **14**(4): 1008-1012

Thornley, C. (2002) Annual summary of outbreaks in New Zealand 2001. *Institute of Environmental Science and Research. Porirua: ESR*

Todar, K. (2005) Todar's online textbook of bacteriology: the genus *Bacillus*. *University of Wisconsin- Madison, Department of bacteriology* URL: http://nsdl.resource/2200/test.20061004104227563T

Twort, F. W., and Lond, L. R. C. P. (1915) An investigation on the nature of ultramicroscopic viruses. *The Lancet* **2**: 1241-1243

Väisänen, O. M., Mentu, J., and Salklnoia-Salonen, M. S. (1991) Bacteria in food packaging paper and board. *Journal of Applied Bacteriology* **71**:130-133

van Netten, P., Perales, I., Van de Moossdijk, A., Curtis, G. D. W., and Mossel. D. A. A. (1989) Liquid and solid selective differential media for the detection and enumeration of *Listeria monocytogene*s. *International Journal of Food Microbiology* **8**(4): 299-316

van Regenmortel, M. H., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., and Lemon, S. M. (eds) (2000) *Virus taxonomy. Classification and Nomenclature of Viruses. Seventh report of the International Committee on Taxonomy of Viruses.* San Diego: Academic Press

Wagner, P. L., and Waldor, M. K. (2002) Bacteriophage control of bacterial virulence. *Infection and Immunity*. **70**:3985-3993

Waldor, M. K., and Mekalanos, J. J. (1996) Lysogenic Conversion by a Filamentour Phage Encoding Cholera Toxin. *Science* **272**(5270): 1910-1914

Waldor, M. K., Friedman, D. I., and Adhya, S. L. (2005) Phages: Their Role in Bacterial Pathogenesis and Biotechnology. ASM Press

Walker, K. (2006) *Use of bacteriophages as novel food additive.* FS06 ANR 490/811 Food Regulation in the United States. Michigan State University

Walter, M. H., and Baker, D. D. (2003) Three *Bacillus anthracis* Bacteriophages from Topsoil. *Current Microbiology* **47**:55-58

Wang, I. N., Dykhuizen, D. E., and Slobodkin, L. B. (1996) The evolution of phage lysis timing. *Evolutionary Ecology* **10**:545–558

Watson, J. D. (1950) The properties of X-ray inactivated bacteriophage. I. Inactivation by direct effect. *Journal of Bacteriology.* **60**: 697-718

Wendlinger, G., Loessner, M. J., and Scherer, S. (1996) Bacteriophage receptors on *Listeria monocytogenes* cells are the N- acetylglucosamine and rhamnose substituents of teichoic acids or the peptidoglycan itself. *Journal of Microbiology* **142**: 985-992

Whichard, J. M., Sriranganathan, N., and Pierson, F. W. (2003) Supression of *Salmonella* growth by wild-type and large plaque variants of bacteriophage Felix 01 in liquid culture and on chicken frankfurters. *Journal of Food Protection* **66**: 220-225

Whichard, K., Morita, M. Fischer, C. R. Yoichi, M., Tanji, Y., and Unno, H. (2003) Coevolution of bacteriophage PP01 and *Escherichia coli* O157:H7 in continuous culture. *Applied Environmental Microbiology* **69**: 170-176

Williamson, S. J., McLaughlin, M. R., and Paul, J. H. (2001) Interaction of the ΦHSIC virus with its host: lysogeny or pseudolysogeny? *Applied and Environmental Microbiology* **67**: 1682-1688

Wong, T. (2007) Environmental Science and Research (ESR) Christchurch Science Centre, personal communication

Wong, T. L., Carey-Smith, G., Hollis, L., and Hudson, J. A. (2005) Microbiological quality of pre-packaged paˆ te’ and ham in New Zealand. *Letter to Applied Microbiology* **41**: 106–111

World Health Organization, January 2002, Fact Sheet #237, Food Safety and Foodborne Illness

Wright, D. J., Chapman, P. A., and Siddons, C. A. (1994) Immunomagnetic separation as a sensitive method for isolating *Escherichia coli* 0157 from food samples. *Epidemiology of Infection* **113**(1): 31-39

Yelton, D. B., and Thorne, C. B. (1970) Transduction in *Bacillus cereus* by Each of Two Bacteriophages. *Journal of Bacteriology* **102**(2):573-579

Yelton, D. B., and Thorne, C. B. (1971) Comparison of *Bacillus cereus* Bacteriophages CP-51 and CP-53. *Journal of Virology* **8**(2): 242-253

Young, R. and Bliisi, U. (1995) Holins: form and function in bacteriophage lysis *FEMS Microbiology Reviews* **17**:191-205

Young, R., Wang, I., and Roof, W. D (2000) Phages will out: strategies of host cell lysis. *Trends in Microbiology* 8(3):120-128

Zink, R., and Loessner, M. J. (1992) Classification of Virulent and Temperate Bacteriophages of *Listeria* spp. on the Basis of Morphology and Protein Analysis. *Applied and Environmental Microbiology* **58**(1): 296-302

Zwietering, M. H., Jongenburger, I., Rombouts, F. M., and van T Riet, K. (1990) Modeling of the Bacterial Growth Curve. *Applied Environmental Microbiology* **56**: 1875-1881

Appendices

A.1 General Materials Required

A.1.1 Salt and Magnesium (SM) Buffer (Frost et al., 1999)

Ingredients:
0.05 M Tris (FW=121.4 = 6.057g/L stilled H_20)
NaCl 5.8 g/L

Supplements:
$MgSO_4$ 2.0 g/L
Gelatin 5 ml/L of 2% w/v solution (0.2g/10ml)

Procedure:

1. Weight Tris, NaCl and $MgSO_4$ and dissolved in distilled water. Adjust pH to 7.5 before autoclaving.
2. Add filter-sterilised (0.22µm) supplement of Gelatin as required

A.1.2 0.1 M $MgSO_4$ Solution

BDH $MgSO_4$ FW = 197.1g/M (100 ml = 0.1 L)
197.1 g/M x 0.1 M = 19.71 g/L
19.71 g/L x 0.1 L = 1.979g
1.979 g of BDH $MgSO_4$ supplement
Dissolved in 100 mL of distilled water.

Autoclave: 121°C for 15 min

A.1.3 50 mM $CaCl_2$ Solution

BDH $CaCl_2$ FW = 110.99g/M (100 ml = 0.1 L)
110.99 g/M x 0.05 M = 5.55 g/L
5.55 g/L x 0.1 L = 0.555 g
0.555 g of BDH $CaCl_2$ supplement
Dissolved in 100 mL of distilled water.

Autoclave: 121°C for 15 min

Components of Invitrogen Luria Broth (LB) Power

NaCl	5 g/l
Peptone 140 (pancreatic digest of casein)	10 g/l
Yeast Extract autolysed low sodium	5 g/l

A.1.4 LB Broth

30 g of Invitrogen LB powder
Dissolved in 1 L of distilled water.
Autoclave: 121°C for 15 min

Final pH: 7.5 +/- 0.2

A.1.5 LB Broth With 15% Glycerol

7.5 g of Invitrogen LB powder
Glycerol 150 ml/L of 15% (w/v) solution
Dissolved in 250 L of distilled water.
Autoclave: 121°C for 15 min

Final pH: 7.5 +/- 0.2

A.1.6 3% Beef extract

Dissolve 3 g of Bacto® beef extract in 75 ml heated distill water
Bring volume up to 100 ml with cold distill water
Adjust pH to 7.5

Autoclave: 121°C for 15 min

A.2 Bacillus Media

Components of Bacto ™ Brain Heart Infusion (BHI)

Calf Brains, Infusion from 200g	7.7 g
Beef heart	9.8 g
Proteose Peptone	10.0g
Dextrose	2.0g
Sodium Phosphage	5.0g

Final pH: 7.4 +/- 0.2

A.2.1 BHI Broth

37g of Bacto ™ BHI powder
Dissolved in 1 L of distilled water.
Autoclave: 121°C for 15 min
Final pH: 7.5 +/- 0.2

A.2.2 BHI Agar (1%)

37g of Bacto ™ BHI powder
Dissolved in 1 L of distilled water
Merck Agar-Agar 15 g
Autoclave: 121°C for 15 min
Final pH: 7.5 +/- 0.2

A.2.3 BHI Soft-Agar Overlay (1%)

Bacto ™ BHI Broth Base 37g
Distilled Water 1 L
Merck Agar-Agar 15 g
Dispense into glass test tubes in 4 ml quantities with lid
Autoclave: 121°C for 15 min
Final pH: 7.5 +/- 0.2

A.2.4 BHI Soft-Agar Overlay (0.8 %)

Bacto ™ BHI Broth Base 37g
Distilled Water 1 L
Merck Agar-Agar12 g
Dispense into glass test tubes in 4 ml quantities with lid
Autoclave: 121°C for 15 min
Final pH: 7.5 +/- 0.2

A.2.5 BHI Soft-Agar Overlay (0.6 %)

Bacto ™ BHI Broth Base 37g
Distilled Water 1 L
Merck Agar-Agar 9 g
Dispense into glass test tubes in 4 ml quantities with lid
Autoclave: 121°C for 15 min
Final pH: 7.5 +/- 0.2

A.2.6 BHI Soft-Agar Overlay (0.4 %)

Bacto ™ BHI Broth Base 37g
Distilled Water 1 L
Merck Agar-Agar 6 g
Dispense into glass test tubes in 4 ml quantities with lid
Autoclave: 121°C for 15 min
Final pH: 7.5 +/- 0.2

Components of Difco™ MYP Agar

Beef extract	1.0 g/L
Peptone	10.0 g/L
D-Mannitol	10.0 g/L
Sodium Chloride	10.0 g/L
Phenol Red	25.0 mg/L
Agar	15.0 g/L

Components of Difco™ Antimicrobic Vial P

(Approximately 30,000 units of polymyxin B per vial)

Aseptically add 5 ml sterile distill water
Rotate in an end-over-end motion to dissolve the contents completely

A.2.7 MYP Agar (plates)

46 g of Difco™ MYP powder
Dissolved in 900 ml distilled water with heat and frequent agitation
Dispense 225 ml into 500 ml flasks
Autoclave: 121°C for 15 min
Cool to 48°C
Aseptically add 12.5 ml Egg Yolk Enrichment 50% and 4.1 ml of rehydrated Antimicrobic Vial P.

Final pH: 7.2 +/- 0.2

A.2.8 Cinderella Instant Mashed Potatoes Mixture

20 g of instant mashed potato
180 ml distill water
Mix well and transferred into 500 ml sterilised bottle

Autoclave: 121°C for 15 min

A.3 Listeria Media

Components of Bacto ™ Tryptic Soy Broth (TSB) Broth:

Pancreatic Digest of Casein	17.0 g
Enzymatic Digest of Soybean Meal	3.0 g
Dextrose	2.5g
Sodium Chloride	5.0g
Dipotassium Phosphate	2.5g

A.3.1 TSB Broth

30g of Bacto ™ TSB powder
Dissolved in 1 L of distilled water.
Autoclave: 121°C for 15 min
Final pH: 7.5 +/- 0.2

A.3.2 TSB Agar (1% agar)

30g of Bacto ™ TSB powder
Merck Agar-Agar 15 g
Dissolved in 1 L of distilled water
Autoclave: 121°C for 15 min
Final pH: 7.5 +/- 0.2

A.3.3 TSB Soft-Agar Overlay (0.5% agar)

30 g Bacto ™ TSB power
Merck Agar-Agar 6 g
Dissolved in 1 L of distilled water
Dispense into glass test tubes in 4 ml quantities with lid
Autoclave: 121°C for 15 min
Final pH: 7.5 +/- 0.2

A.3.4 TSB Soft-Agar Overlay (0.5 % agarose)

30 g Bacto ™ TSB power
6 g of Invitrogen UltraPure™ agarose
Dissolved in 1 L of distilled water
Dispense into glass test tubes in 4 ml quantities with lid
Autoclave: 121°C for 15 min
Final pH: 7.5 +/- 0.2

A.3.5 Fraser Broth

Components of Oxoid Fraser Broth Base:

Proteose peptone	5.0 g/L
Tryptone	5.0 g/L
Lab-Lemco power	5.0 g/L
Yeast extracts	5.0 g/L
Sodium chloride	20.0 g/L
Di-sodium hydrogen phosphate	1.35 g/L
Potassium dihydrogen phosphate	1.35 g/L
Aesculin	1.0 g/L
Lithium chloride	1.0 g/L

28.7g of Fraser broth base
Dissolved in 500 ml distilled water
Autoclave: 121°C for 15 min

Final pH: 7.2 +/- 0.2

Components of Oxoid Fraser Supplement (for 500 ml of Fraser broth)

Ferric ammonium citrate	0.25g
Nalidixic acid	10.0mg
Acriflavine hydrochloride	12.5 mg

A.3.6 Oxoid Fraser Supplement

Aseptically add 5 ml of ethanol/sterile water (1:1)
Rotate in an end-over-end motion to dissolve the contents completely
Distribute into sterile container and stored at 4°C

Components of Oxoid Palcam Agar Base

Columbia blood agar base	39.0 g/L

Yeast extracts	3.0 g/L
Glucose	0.5 g/L
Aesculin	0.8 g/L
Ferric ammonium citrate	0.5 g/L
Mannitol	10.0 g/L
Phenol red	0.08 g/L
Lithium chloride	15.0 g/L

Components of Oxoid PALCAM Selective Supplement (Supplement for 500 mL)

Polymixin B	5 mg
Acriflavine hydrochloride	2.5 mg
Ceftazidime	10 mg

Reconstituted with 2 ml of sterile distilled water

A. 3.7 PALCAM Agar Plate

34.5 g of Oxoid PALCAM agar base
Dissolve in 500 ml of distilled water with heat until gently boiled
Autoclave: 121°C for 15 min
Cool to 48°C
Aseptically add contents of one vial of PALCAM Selective Supplement
Mixed well and pour into sterile Petri dishes

Final pH: 7.2 +/- 0.2

A.4 Immunomagnetic Separation (IMS) Medias

A.4.1 Components of Oxoid Phosphate Buffered Saline (PBS) Tablet

Na2HSO4 (Disodium hydrogen phosphage)	1.15 g
KH2SO4	0.20 g
NaCl	8.00 g
KCl	0.20 g

1 tablet dissolved in 100 ml of distilled water

Autoclave: 121°C for 15 min

A.4.2 Sigma Bovine Serum Albumin (BSA)

1 g of BSA dissolved in 20 ml of distilled water

Filter sterilise with 0.22µl filter

A.4.3 PBS/BSA

2 ml of BSA added into 100 mL PBS to make up PBS containing 0.1 % of BSA.

A.5 Transmission Electron Microscope (TEM) Medias

A.5.1 0.1 M Ammonium Acetate

Scharlau Ammonium acetate FW= 77.08 g/M
77.08 g/M x 0.1 M = 7.708 g/L
7.708 g of A Scharlau mmonium acetate
Dissolved in 1L of distilled water

Autoclave: 121°C for 15 min

A.5.2 Negative Stain (1% PTA)

0.2 g of phosphotungastic acid
Dissolved in 15 ml of double distilled water
Adjust pH to 6.5 with dilute NaOH
Make volume up to 20 ml with distilled water

Filter sterilise with 0.22µl filter and store at 4℃

A.5.3 Coating Carbon-Coated 300 Mesh Copper Grids

Expose carbon-coated 300 mesh copper grids to air plasma at 5 x 10^{-1} mbar in an Edwards 306A coating unit fitted with glow-discharge electrodes for 1 min.

A. 6 DNA Extraction and Gel Media

A.6.1 0.5 x TBE Buffer

60.5 g Tris
31 g Boric Acid
3.7 g EDTA
Dissolved in 800 ml of distilled water
Bring volume up to 1L with distilled water

Autoclave: 121°C for 15 min
(This is 5 x TBE buffer, kept in fridge and a 1:10 dilution was made with steriled distill water to make 0.5 x TBE buffer as required)

A.6.2 1% gel (1 cm thick gel)

0.8 g of agarose
80 ml 0.5 x TBE buffer
Weight the conical flask before and after heating
Dissolved in conical flask with microwave
Add steriled mili-Q water to bring up the volume to original weight
Cool at 48°C for 10 min
Pour the gel in rack with walls and leave it for 15 min in room temperature

A.6.3 0.5 M Ethylenediaminetetra-Acetic Acid (EDTA)

BDH EDTA FW = 292.25 g/M
292.25 g/M x 0.5 M = 146.125 g/L
146.125 g of BDH EDTA
Dissolved in 1L of distilled water

Autoclave: 121°C for 15 min

A.6.4 20% Sodium Dodecyl Sulphate (SDS)

20 g of BDH SDS power
Dissolved in 800 ml of distilled water
Bring volume up to 1L with distilled water

Autoclave: 121°C for 15 min
Final pH = 7.5

A.6.5 3M Sodium Acetate

LabServ™ Sodium acetate FW = 136.08 g/M

136.08 g/M x 3 M = 408.24 g/L (100 ml = 0.1 L)
408.24 x 0.1 = 40.824 g
40.824 g of LabServ™ Sodium acetate
Dissolved in 100 ml of distilled water
Check and adjust pH to 7.5

Autoclave: 121°C for 15 min

A.6.6 70 % Ethanol

700 ml of BDH 100% ethanol
Diluted with 300 ml of distill water

A.6.7 50 mM Tris

Probiogen Tris FW = 121.14 g/M (100 ml = 0.1 L)
121.14 g/M x 0.05 M = 6.057 g/L
6.057 g/L x 0.1 L = 0.657 g
0. 657 g of Probiogen Tris
Dissolved in 100 ml of stilled water
Check and adjust pH to 7.5

Autoclave: 121°C for 15 min

A. 7 *Poster to be presented at the Edinburgh International Phage Conference, 26-29th July 2008 and the International Association for Food Protection Conference, Columbus, Ohio, 3-6 August 2008*

Isolation of Bacteriophages Infecting Gram-Positive Foodborne Pathogens

Wan-Jing Lee[1,2], J. ANDREW HUDSON[1], Jack Heinemann[2], Craig Billington[1] and Lynn McIntyre[1]

[1]Food Safety Programme, ESR Ltd, PO Box 29-181, Christchurch New Zealand
[2]School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

Introduction

There are now several publications reporting the inactivation of foodborne pathogens using bacteriophages (phages), and most of this work has focused on Gram-negative organisms. However there has also been interest in controlling *Listeria monocytogenes*, with at least two commercial phage-containing products having GRAS status for food use, and there have been some reports of control of *Staphylococcus aureus*. Our group has isolated phages infecting *Salmonella*, *Campylobacter* and *Escherichia coli* O157:H7, but we have only recently sought phages infecting the Gram-positive bacteria *L. monocytogenes* and *Bacillus cereus*. We are exploring the possibilities of using either phages or phage-based techniques to control these pathogens in foods. We report here the isolation and characterisation of some phages, as well as showing the control of *B. cereus* in reconstituted mashed potato.

Materials and Methods

Isolation of phages from a variety of environmental and food sources was attempted by standard methods using agar overlays. Plaques were than isolated, purified and made as high titre stocks. Phage ultrastructure was determined by electron microscopy and genome size estimates from restriction digests of isolated DNA. Host ranges were ascertained using the spot test and single-step growth curves used to obtain growth cycle data.

Inactivation of bacterial host in food was measured in sterile reconstituted mashed potato formulated to a more fluid than normal consistency to allow mixing dispensing etc. Samples were periodically withdrawn and host cells enumerated on Brain Heart Infusion agar plates.

Results

Phages Infecting B. cereus

Two phage isolates (FWLBc1 and FWLBc2) which appeared to produce different plaque types on agar overlays were obtained from soil.

The morphology of these phages is shown in Figure 1 and the overall dimensions in Table 1. All phages had contractile tail sheaths. The host ranges of the two phages are shown in Table 2. In some cases inhibition zones that formed only at the highest titre of phages added. That they did not form with 10-100 fold dilutions of phages may indicate lysis from without and not "productive infection".

In broth culture, addition of phages resulted in complete host lysis as measured by optical density (e.g. Figure 2). FWLBc2 had a burst size of 300 (+/- 8) pfu (Figure 3) and a latent period of 84 (+/- 2) min. The fraction of phage adsorbed within 5 min was 0.98. FWLBc1 behaved similarly.

Genome size estimates derive from phage DNA digested using the restriction enzymes *Hin*6I, *Sap*I, *Dra*I, and *Taq*I. The DNA of neither phage DNA was cleaved by *Bsp*143I. The other enzymes generated similar restriction patterns were very similar for both phages (Figure 4). Both phages had genome sizes of approximately 134 kb. When all of these data are considered it is likely that the two phage isolates are very closely related or identical.

When phages were added to *B. cereus* in sterile mashed potato and incubated at 37°C at a phage:host ratio of 1:1 there was only a slight reduction in the pathogen compared to the uninfected control. However, when this ratio was increased to 1,000:1 (approximately 10^9 pfu/ml) the decline in pathogen numbers was rapid and sustained throughout the course of the experiment (Figure 5).

Phages infecting L. monocytogenes

Five isolates were obtained from sheep faeces and, according to morphological and host range data (Tables 1 and 3), probably represent three different phages (FWLLm1, FWLLm3 and FWLLm5). Preliminary kinetic experiments in broth (Figure 6) showed that the lysis of host *L. monocytogenes* 2000/47 occurred in a dose-dependent manner. These data were typical of all five phage isolates.

Table 1. Physical characteristics of phages based on TEM.

Physical characteristics	FWLBc1	FWLBc2	FWLLm1	FWLLm3	FWLLm5
Head diameter, nm (average)	97 ± 5	96 ± 6	98 ± 9	108 ± 11	100 ± 7
Tail length, nm (average)	210 ± 25	219 ± 10	222 ± 3.5	209 ± 5	209 ± 8
Sheath, contracted, diameter, nm (average)	23 ± 4	27± 4	26 ± 3	25 ± 1	23 ± 4
Tail tube, diameter, nm (average)	10 ± 3	10 ± 2	8 ± 1	9.3 ± 3	8 ± 1

Table 2. Host range of *Bacillus* phages FWLBc1 and FWLBc2

Indicator hosts	FWLBc1	FWLBc2
A2 (*B. cereus* from potato)	+	+
D2 (*B. cereus* from potato)	+	+
B. cereus (NCTC 11143)	-	LWO
B. cereus (NCTC 11145)	-	LWO
B. cereus (NZRM5)	LWO	LWO
B. thuringiensis (NZRM2981)	-	-
B. thuringiensis (DSM 2046)	+	+
B. licheniformis (DSM 603)	-	LWO
B. subtilis (NTCT 3610)	-	-
B. mycoides (ATCC 6462)	LWO	LWO
B. mycoides (from raw mussel)	+	+
B. megaterium (NCTC 10343)	-	-

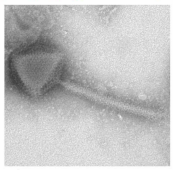
+ clear plaques, LWO clearing possibly due to lysis from without

Table 3. Host range of L. monocytogenes phages

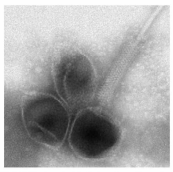
Indicator hosts	FWLLm1	FWLLm3	FWLLm5
L. monocytogenes NZRM 44	+	+	+
L. monocytogenes NZRM 2592	+	+	+
L. monocytogenes NZRM 2594	+	+	+
L. monocytogenes NZRM 3370	+	LWO	+
L. monocytogenes NZRM 3449	LWO	+	LWO
L. monocytogenes NZRM 3450	LWO	+	LWO
L. monocytogenes 3009	+	LWO	+
L. monocytogenes 2000/47	+	+	+
L. grayi	-	LWO	LWO
L. welshimeri	+	+	+
L. ivanovii	+	LWO	LWO
L. innocua	LWO	+	-

+ clear plaques, LWO clearing possibly due to lysis from without

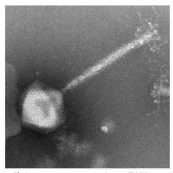
Figure 1. Electron Micrographs of Phages Isolated (and one *L. monocytogenes* typing phage). Dimensions are given in Table 1.



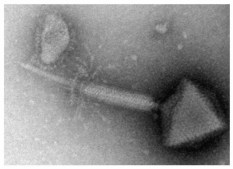
A) FWLBc1 Uncontracted tail sheath.



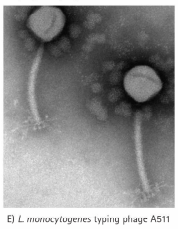
B) FWLBc2 Contracted tail sheath and empty head.



C) *L. monocytogenes* phage FWLLm5



D) *L. monocytogenes* phage FWLLm1 with contracted tail



E) *L. monocytogenes* typing phage A511

Figure 2. Lysis of *B. cereus* by phage FWLBc2.

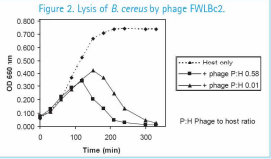


Figure 3. Single step growth of Bacillus phage FWLBc2 on host D2 at 37°C.

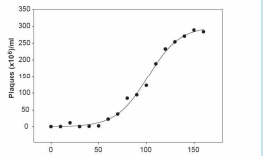


Figure 4. Digests of DNA from *Bacillus* phages and *L. monocytogenes* phage A511.

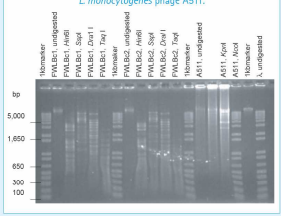


Figure 5. Reduction in counts of *B. cereus* incubated at 37°C in mashed potato. Bc1 and Bc2 refer to the two phages FWLBc1 and FWLBc2. Note the rapid >4 log10 reduction in numbers with phages added at approx. 10^9 pfu/ml.

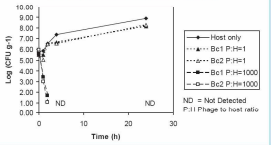
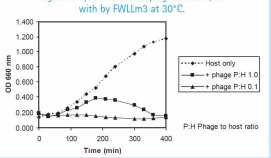


Figure 6. Lysis of *L. monocytogenes* 2000/47 with by FWLLm3 at 30°C.



Discussion

The host ranges of the *Bacillus* phages suggests that they will not be useful individually as they did not lyse all isolates of *B. cereus* tested. However, they did significantly reduce pathogen numbers in mashed potato and so could contribute to an eventual "cocktail" of phages active against this species and perhaps other significant species.

The *L. monocytogenes* phages are less well characterised but their host range is broad enough to indicate promise as biocontrol agents. They were able to infect *L. monocytogenes* 2000/47, a strain which recurs in New Zealand clinical cases, while the typing phage A511 did not. The host was inactivated rapidly to a low optical density in vitro, showing the lytic potential of the phages.

The data we have obtained for a range of phages suggests that they show promise as a means of controlling foodborne pathogens, as has been recognised by the recent appearance of phage products commercially.

Acknowledgements

We than ESR for the post-graduate fellowship (W-J.L.). This work was done as part of a contract for the New Zealand Foundation for Research, Science and Technology (contract no. CO3X0201). The Electron Microscopy Unit at the University of Otago is thanked for producing the electron micrographs. Steve Flint from Fonterra Palmerston North provided phage A511.

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Appendices

Acknowledgements

I thank the Institute of Environmental Science and Research (ESR) Ltd for their financial support through the scholarship and The New Zealand Foundation for Research, and Science and Technology for the materials used in the project. This thesis would not have been possible without the opportunity given by ESR Christchurch Science Centre. I would like to give special thanks to my supervisors: Assoc. Prof. Jack Heinemann, School of Biological Sciences of the University of Canterbury, and Dr. Andrew Hudson, Food Safety Programme of the Institute of Environmental Science and Research Ltd.; for their time, thoughts, encouragement, advices, and guardianship to a student without much microbiology background and lab skills.

Much thanks are also given to all members of the phage group: Craig Billington, Lynn McIntyre, Teresa Bigwood, Aruni Premaratne, and Stephanie Brandt and previous employee, Salim Ismail; for all their enthusiasm, creativity and, most importantly, the encouragement and assistance, when I messed things up, and their willingness to share their knowledge. To all the ESR staff, I extend my warmest appreciation for all the helps through the good and bad times and, especially, for their welcoming and embracing; I recognise the tremendous expense in hosting a student, and am grateful. It was also great to have all the members in Jack's group meeting, especially Brigitta Kurenbach and Sophie Walker for their kind supports and suggestions through out my big time in Masters presentation.

I need to thank The Electron Microscopy Unit at the University of Otago for producing the electron micrographs, Steve Flint from Fonterra Palmerston North for providing phage A511 and *Bacillus* species, and TeckLok Wong for providing *B. mycoides* for host range testing.

The participations and assistances of sample collection provided by the staff members in PPCS Ltd, Belfast Centre, Tegel Food Ltd, Hornby Centre, and ESR were much appreciated as well.

I want to convey my appreciation to my family and all my friends who encouraged and prayed for me during this period of time. I would like to make a special thank to my dad, because he never seems to be able to get hold of his daughter during the studying period. I am also grateful to Jessie and Allen Hou for their support and for sharing everything with me, including their office and the stresses, and difficulties during our time as a Masters candidate. I want to acknowledge the special friendship of the group of people who meet up at Eric Wong's place very often, for their encouragement, understanding and support during my time of depression and for the time-out lunches and coffees when I had no energy for anything else but fun. I really appreciated Mike for his attempts in correcting my English, and his kindness in providing a very warm place for me to study. Lastly but most importantly, I would like to give all the glory to God, who has been merciful and grateful all the way, and guided, and blessed me through all my very difficult times. I would not have even continued the Post-graduate study without Him and am thankful for all the blessings and encouragements He gave me through all the people around me.